Antifungal Activities of Metergoline, Purpurin and Baicalein on *Candida* Species

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ABSTRACT

Candidiasis has become a serious infection with very high mortality and morbidity in the world if not providing effective treatments. However, due to clinical limitation and resistance of the current antifungal agents, there is an urgent need to search for novel antifungals. In this study, after screening a compound library (n=400) for antifungal activity, three members (metergoline, purpurin and baicalein) were chosen for further study. Their antifungal characteristics and the antifungal mechanisms were investigated.

Metergoline, a serotonin receptor antagonist, was found to have potent antifungal activity against the intrinsically fluconazole-resistant human fungal pathogen *Candida krusei*. The minimal inhibitory concentration and minimal fungicidal concentration of metergoline against *C. krusei* were 4 μg/ml and 8 μg/ml respectively. Metergoline induced post-antifungal effect. Significant synergism was found in combination of metergoline with amphotericin B by a checkerboard assay, which may be due to the perturbation of cell permeability and increase in the intracellular accumulation of antifungal agents. Metergoline also inhibited extracellular phospholipase production in *C. krusei*. To gain insights into the mechanisms, intracellular changes that accompany apoptosis were examined by flow cytometry and spectrophotometry. The results showed an increase in the level of reactive oxygen species, depolarization of mitochondrial membrane potential, phosphatidylserine externalization, and positive terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labelling in the

metergoline-treated *C. krusei*. Taken together, we conclude that metergoline may promote apoptosis in *C. krusei* through reactive oxygen species production and perturbation in mitochondrial homeostasis, implying its antifungal potential to treat candidiasis.

The antifungal activity of purpurin, a natural red anthraquinone pigment in madder root (*Rubia tinctorum* L.), was evaluated against *Candida* isolates by a broth microdilution assay. The minimal inhibitory concentrations of purpurin against *Candida* species isolates were 1.28-5.12 μg/ml. Mechanistic studies indicated that purpurin inhibited energy-dependent efflux pumps of *Candida* isolates. Furthermore, purpurin demonstrated a depolarization of mitochondrial membrane potential, suggesting a possible linkage of the antifungal mechanism of purpurin to *Candida* apoptosis.

Baicalein is known to be a potent antifungal agent and induces programmed cell death in *Candida albicans*. In the present study, we found that baicalein also inhibited the growth of *C. krusei* isolates. The minimal inhibitory concentrations of baicalein against eight *C. krusei* isolates were 1.35–2.70 μg/ml. One-hour exposure to baicalein elicited a consistent and moderate post-antifungal effect on the *C. krusei* isolates. Further flow cytometric study demonstrated a depolarization of mitochondrial membrane potential. However, both the levels of reactive oxygen species and DNA fragmentation were not significantly changed after baicalein treatment in *C. krusei*. It can be concluded that the antifungal activity of baicalein was mitochondria-dependent in both *C. krusei* and *C. albicans*, but the antifungal mechanism was different. Reactive oxygen species may not play a direct role and

baicalein does not initiate programmed cell death or apoptosis in *C. krusei*. The structure-activity relationship study showed that the three hydroxyl groups in baicalein were essential for its antifungal potency.

論文摘要

念珠菌感染如果不得到有效的治療，其死亡率很高。然而目前臨床上可提供的抗真菌藥種類有限，且毒副作用和耐藥性也限制了其進一步的應用，因此急需尋找新型高效的抗真菌藥。通過對一個化合物庫抗真菌活性篩選，我們發現了其中三個化合物具有抗真菌作用：麥角苷酯， 紅紫素和黃芩苷 ，並對其抗真菌活性及作用機制進行了研究。

麥角苷酯是一種五羥色氨受體拮抗劑，其對內在性氟康唑耐藥的人源性病原體 *Candida krusei* 具有強效的抑制作用。麥角苷酯的最小抑菌濃度和最小殺菌濃度分別爲 4 μg/ml 和 8 μg/ml。麥角苷酯與兩性黴素 B 有顯著的協同作用，此協同作用可能是由於麥角苷酯增加了真菌細胞的通透性，從而增加了細胞內的藥物累積。麥角苷酯也抑制 *C. krusei* 細胞外磷脂酶的分泌。作用機制研究表明：*C. krusei* 經麥角苷酯作用後，活性氧成份顯著增加，線粒體膜電位降低，磷脂醯絲氨酸外翻，TUNEL 標記後細胞呈陽性。因此，我們認爲麥角苷酯通過產生活性氧成份及幹擾線粒體穩定性而促使 *C. krusei* 產生凋亡。

一種存在於茜草根部的天然蒽醌色素，紅紫素，具有廣譜的抗念珠菌活性。紅紫素的最小抑菌濃度在 1.28 μg/ml 和 5.12 μg/ml 之間。羅丹明 6G 外排實驗研究表明，紅紫素能夠抑制念珠菌的能量依賴性外排泵活性。而且紅紫素能降低線粒體膜電位，這可能是紅紫素的抗念珠菌機制。

黃芩苷作爲一個有效的抗真菌活性化合物，能夠誘導 *C. albicans* 產生程式性細胞死亡。本文研究發現，黃芩苷對 *C. krusei* 也有抑制作用。對 *C. krusei* 的最小抑菌濃度在 1.35 μg/ml 和 2.70 μg/ml 之間。黃芩苷对 *C .krusei* 有輕度的抗菌後效應。流氏細胞術研究表明，黃芩苷降低 *C. krusei* 的線粒體膜電位。然而，*C. krusei* 中活性氧成份和 DNA 片斷卻沒有顯著改變。我們推測，對於 *C. krusei* 和 *C. albicans*，黃芩苷的抗菌活性可能都是線粒體依賴性的，然而作用機制存在差別。在 *C. krusei* 中，活性氧成份可能不起直接作用，黃芩苷不會誘導細胞程式性死亡。同時結構功能關係研究表明，黃芩苷上的三個羥基在黃芩苷的抗真菌作用中是不可缺少的。

PUBLICATIONS

Kang K, Wong KS, Jayampath SC, Samaranayake LP, Fong WP, Tsang PWK. *In vitro* synergistic effects of metergoline and antifungal agents against *Candida krusei*. *Mycoses*. 2009, doi:10.1111/j.1439-0507.2009.01747.x.

Kang K, Fong WP, Tsang PWK. Novel antifungal activity of purpurin against *Candida* species *in vitro*. *Medical Mycology*. 2010, doi: 10.3109/13693781003739351.

Kang K, Fong WP, Tsang PWK. Antifungal activity of baicalein against *Candida krusei* does not invole apoptosis. *Mycopathologia*. 2010, doi: 10.1007/s11046-010-9341-2.

Kang K, Samaranayake LP, Fong WP, Tsang WK. *In vitro* synergistic effects of metergoline and antifungal agents against *Candida krusei*. The 17th Congress of The International Society for Human and Animal Mycology: Meeting Abstract, Tokyo, Japan, May 2009.

Kang K, Fong WP, Tsang WK. Mechanistic studies on the metegoline-induced death on *Candida krusei*. The 2nd International Conference on Drug Discovery & Therapy: Meeting Abstract, Dubai, UAE, February 2010.

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ABBREVIATIONS

ABC	ATP-Binding Cassette
AIDS	Acquired Immune Deficiency Syndrome
AMB	Amphotericin B
ATCC	American Type Culture Collection
ATP	Adenosine-triphosphate
CLSI	Clinical and Laboratory Standards Institute
DCFDA	2',7'-Dichlorofluorescin Diacetate
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
FBS	Fetal Bovine Serum
FICI	Fractional Inhibitory Concentration Index
FLC	Fluconazole
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic Acid
JC-1	5,5′,6,6′-Tetrachloro-1,1′,3,3′-Tetraethyl-Imidacarbocyanine Iodide
MDR	Multidrug Resistance
MIC	Minimal Inhibitory Concentration
MFC	Minimal Fungicidal Concentration
MMP	Mitochondrial Membrane Potential
MRR	Multidrug Resistance Regulator
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NMR	Nuclear Magnetic Resonance
OD	Optical Density

PAFE	Post-Antifungal Effect
PBS	Phosphate Buffered Saline
PI	Propidium Iodide
PS	Phosphatidylserine
Pz	Phospholipase production
R6G	Rhodamine 6G
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SD	Standard Deviation
SEM	Scanning Electron Microscopy
TAC	T ranscriptional Activator of CDR Genes
TCM	T raditional Chinese Medicine
TUNEL	Terminal Deoxynucleotidyltransferase-Mediated dUTP-Biotin Nick Labeling
XTT	2,3-Bis(2-Methoxy-4-Nitro-5-Sulfophenyl)-5-[(Phenylamino) carbonyl]-2H-Tetrazolium Hydroxide

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Chapter 1

Introduction

1.1 *Candida* species and candidiasis

Candida species is one of the most prevalent human fungal pathogens, existing as commensal on the skin and mucosal surfaces of oral cavity, gastrointestinal tract and urogenital system in immunoproficient individuals. They are opportunistic and can cause infections when the host immunosystem is compromised such as suffering from AIDS, cancer or organ transplantations. Candidiasis is ranked the fourth in the leading causes of nosocomial infections (Arikan, 2007; Corner and Magee, 1997). More than 90% candidiasis is caused by *C. albicans*. Recently there is an increasing trend of candidiasis arising from non-*albicans* spp. such as *krusei*, *tropicalis*, *glabrata etc* (Krcmery and Barnes, 2002; Drago *et al.*, 2004; MacFarlane and Samaranayake, 1990). Recurrent mild superficial infection such as thrush is not fatal, but the high morbidity and mortality of deep-seated and disseminated candidiasis can be life-threatening.

1.1.1 *Candida albicans*

C. albicans is well-known and classified as one of the most prevalent human fungal pathogens. It colonizes as a commensal in host under normal circumstances. However, when the human immunosystem is compromised, *C. albicans* becomes an

invasive pathogen and patients are highly susceptible to *C. albicans* infections (Arikan, 2007; Corner and Magee, 1997; Kabir and Hussain, 2009). *C. albicans* is dimorphic which exists in either planktonic entity or filamentous, penetrative hyphal form. The latter has higher virulent activity for adherence and invasion of the host to cause an infection. Mild superficial candidiasis such as thrush is common and not fatal, but deep-seated and systemic infections usually result in high morbidity and mortality (Bennett, 2009).

1.1.2 non-*albicans Candida* species

The most common non-*albicans Candida* species include *C. krusei*, *C. tropicalis*, *C. glabrata* (formerly *Torulopsis glabrata*), *C. parasilosis* and *C. kefyr*, which totally represent about one-half of all *Candida* spp. isolated from blood cultures. Clinically, there is special concern to non-*albicans Candida* for two reasons. The first is the virulence activity of these species, mainly in the immunocompromised host, leading to high mortality. The second is the occurrence of resistance to currently available antifungal drugs. These factors constitute a serious clinical problem. Therefore, antifungal susceptibility testing and the development of new antifungal drugs are mandatory to treat candidiasis caused by non-*albicans Candida* species (Krcmery and Barnes, 2002; Wingard *et al.*, 1995; Abi Said *et al.*, 1997).

C. krusei is notorious in the clinical setting due to its inherent resistance to fluconazole (FLC), and many of the infections are caused by the prophylactic or therapeutic use of this antifungal agent (Abbas *et al.*, 2000). The FLC resistant

mechanisms may be due to the overexpression of one or more drug efflux pumps such as ATP binding cassette transporter, or diminished sensitivity of the target enzyme, cytochrome P450 sterol 14α-demethylase, to inhibition by an azole antifungal agent (Katiyar and Edlind, 2001; Takashi *et al.*, 2003). *C. krusei* is rarely detected in candidemia overall but is more common among cancer patients and even more frequent in neutropenic patients with hematological malignancies (Conen *et al.*, 2008). In general, *C. krusei* infections accounts for 2% of candidemia, increasing to 5-10% in cancer patients and 24% in neutropenic patients with underlying hematological malignancies (Marchetti *et al.*, 2004). Most seriously, highest mortality rate (30-60%) has been observed in candidal infections in association with *C. krusei* (Muñoz *et al.*, 2005), which might be related to its multidrug resistant traits.

1.1.3 Biofilm formed by *Candida* species

In addition to exist as planktonic cells, *Candida* species can also encase within polysaccharide-rich extracellular matrix to form biofilm. Fungal biofilm is the most serious clinical problem associated with significant rate of mortality which is mainly caused by *Candida* species (Fig. 1.1). Biofilm can act as reservoirs to provide safe shelter for persistent sources of infection (Ramage *et al.*, 2009). As fungi are eukaryotic cells and they are more complex than bacteria, it is difficult to diagnose and treat these infections. Generally, biofilm can be formed by pathogens on the surfaces of devices in most device-related infections in nosocomial infections in medical intensive care units. Reports have shown that nearly 86% of nosocomial

pneumonia and 95% of urinary tract infections are linked with mechanical ventilation and urinary catheters, respectively (Richards *et al.*, 1999). Formation of biofilm by *Candida* species is diverse, such as cardiovascular devices, joint devices, central venous catheters, dialysis access devices, urinary catheters, dentures and ocular implants (Kojic and Darouiche, 2004; Lane and Mattay, 2002). Governed by complex molecular events, fungal biofilm has developed from adhesion, colonization, maturation and dispersal phases.

Fungal biofilm assumes great significance in the clinical context because it is related to resistance against most antifungal agents (Kuhn and Ghannoum, 2004; Stewart, 2002; Douglas, 2003). Reports have shown that biofilm are 1000-fold less susceptible to antifungal therapy than planktonic cells in a variety of *in vivo* and *in vitro* biofilm models (Di Bonaventura *et al.*, 2006; Ramage *et al.*, 2001). A lot of mechanisms have been elucidated for the antifungal resistance of planktonic fungal cells, such as over-expression of membrane-localized drug efflux pumps, changes in membrane sterol composition and alteration in drugs targets (Ghannoum *et al.*, 1999; Prasad and Kapoor, 1995; White *et al.*, 1998). In contrast, the mechanisms of antifungal resistance of biofilm have not been fully investigated. Several factors may contribute to the resistance, including the role of extracellular matrix, the physiological state of fungal cells, overexpression of drug efflux pumps, limited drug penetration and alteration of sterol composition (Al-Fattani and Douglas, 2004; Keren *et al.*, 2004; Hentzer *et al.*, 2001; Mukherjee *et al.*, 2003; Xu *et al.*, 2000; Pierce *et al.*, 2008).

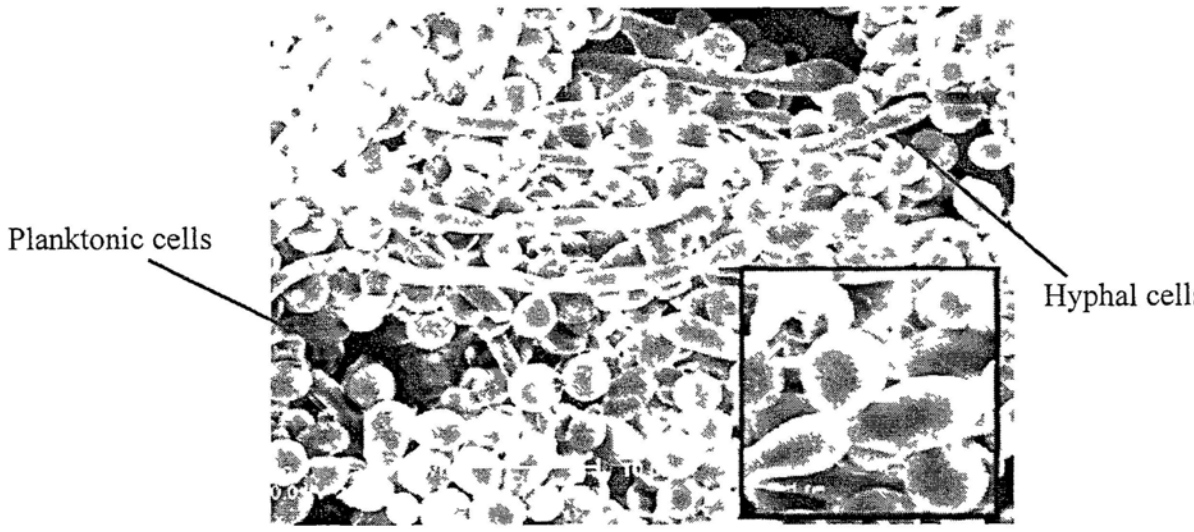


Fig. 1.1 SEM images showing the profile of biofilm (Thein *et al* , 2007).

1.2 Antifungal agents to treat candidiasis

Chemotherapy is the most common method to treat Candidiasis. According to the action effect and different target molecules, current antifungal agents can be classified into two categories: fungicidal and fungistatic. Fungicidal agents include polyenes and echinocandins Fungistatic agents include azoles and DNA analogues.

1.2.1 Polyenes

Polyenes, such as amphotericin B (AMB) and nystatin (Fig 1.2 & Fig. 1.3), bind ergosterol and interact with ergosterol formation, resulting in an increase in membrane permeability and cell death. However, serious side effects such as nephrotoxicity and infusion-related complication are associated with AMB (Olson *et*

al., 2006).

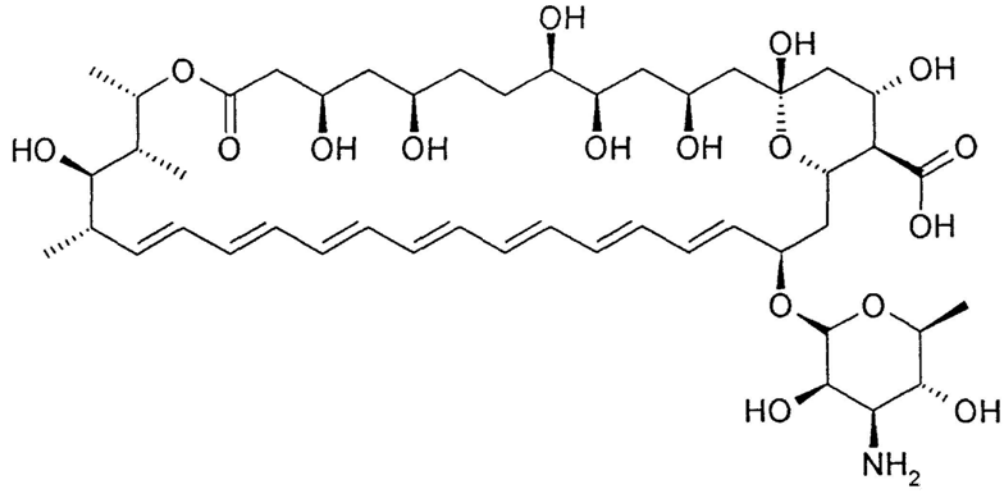


Fig. 1.2 Structure of AMB

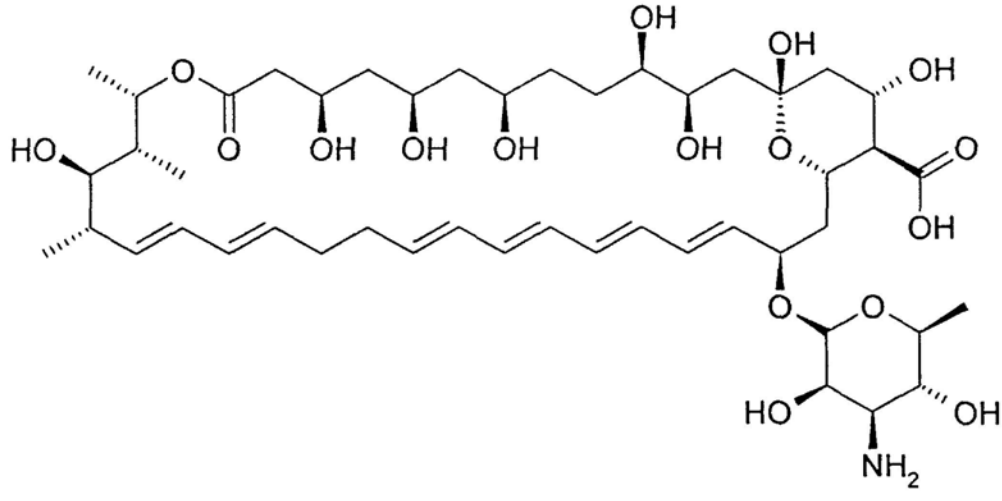


Fig. 1.3 Structure of nystatin

1.2.2 Echinocandins

Echinocandins, such as anidulafungin and caspofungin (Fig. 1.4 & Fig. 1.5), target on the fungal cell wall and inhibit the fungal β-1,3-glucan synthase complex, which lead to the depletion of fungal cell wall and osmotic instability. Due to the lack of cell wall in mammalian cells, this type of antifungal agents has higher selectivity and

lower toxicity than others. However, despite comprehensive efforts, in general, this lipopeptide class of antifungal agents has very poor oral bioavailability, which as a result severely restricts the usage of these effective antifungal agents. Pharmaceutical companies have failed to identify new β-1,3-glucan synthase inhibitors with improved oral bioavailability after an exhaustive screening of chemical and natural product libraries (Denning, 2002; Onishi *et al.*, 2000).

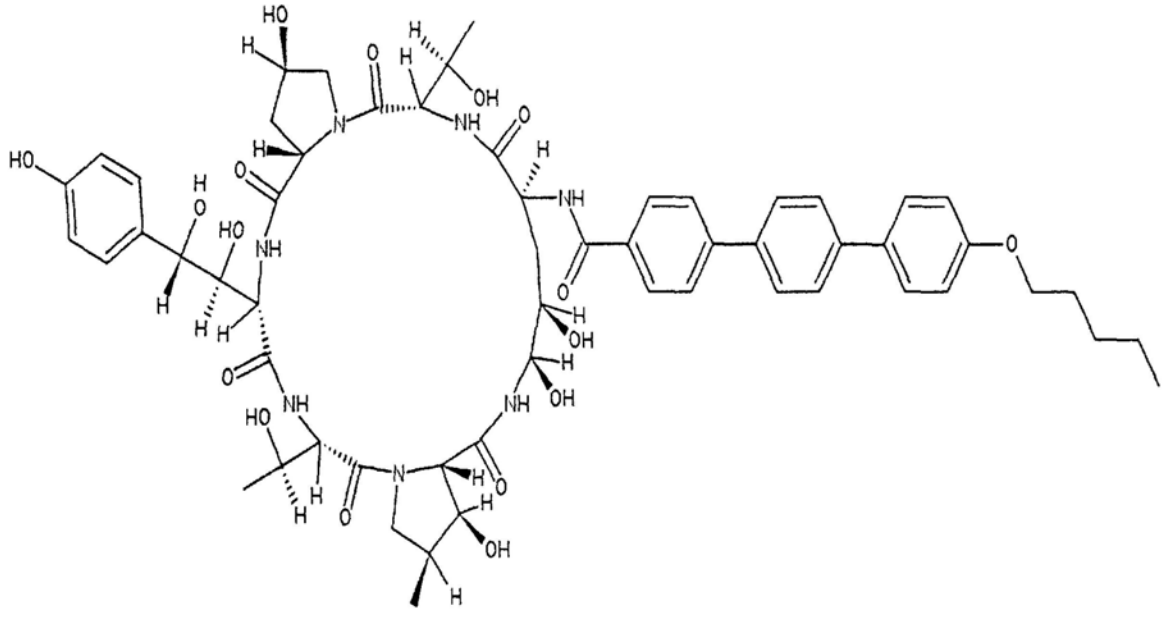


Fig. 1.4 Structure of anidulafungin

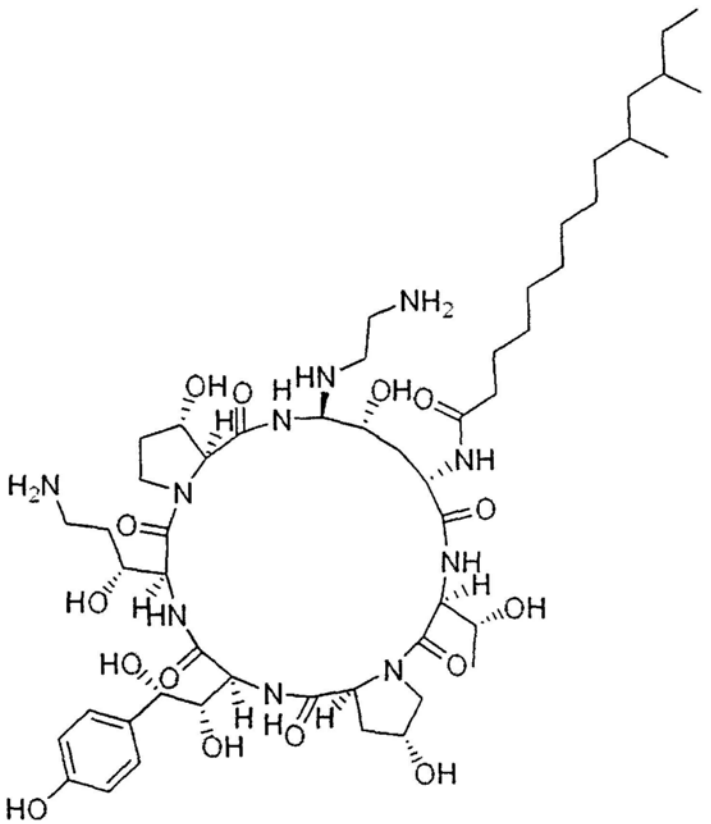


Fig. 1.5 Structure of caspofungin

1.2.3 Azoles

Azoles, known for FLC and with the newest azoles being voriconazole and posaconazole (Fig. 1.6-Fig. 1.8), interfere with lanosterol demethylase and inhibit ergosterol biosynthesis to alter membrane-associated functions. Although the azole antifungals have minimal side-effects and are orally bioavailable, they are not fungicidal and thus require long-term therapeutic regimens. Long-term treatment with these fungistatic drugs, particularly in immunodeficient patients, appears to be the choice for fungal infections caused by innately resistant strains. Also there has been a striking rise in the incidence of acquired azole resistance in clinical isolates (Joon *et al.*, 2007).

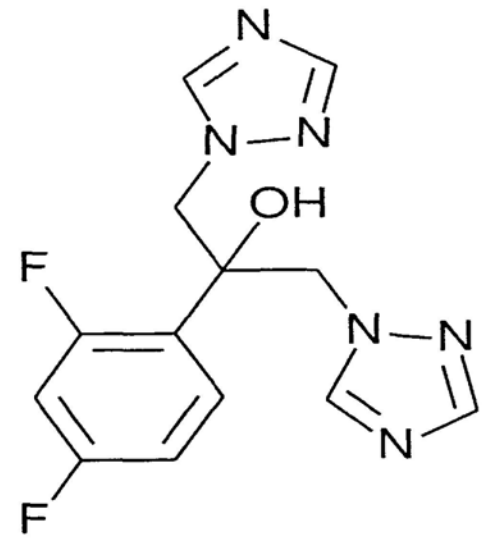


Fig. 1.6 Structure of fluconazole

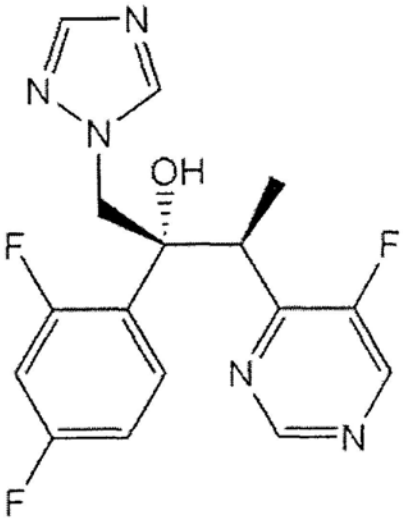


Fig. 1.7 Stucture of voriconazole

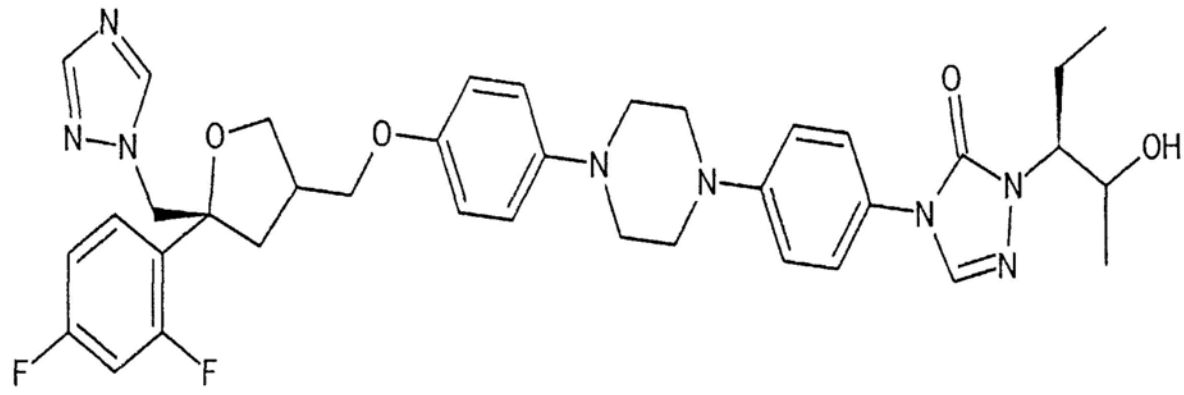


Fig. 1.8 Stucture of posaconazole

1.2.4 DNA analogues

DNA analogues, such as flucytosine (Fig. 1.9), inhibit thymidylate synthase and induce aberrant RNA biosynthesis, but have problems with intrinsic and acquired drug resistance and therefore have limited applications (Fleck *et al.*, 2007).

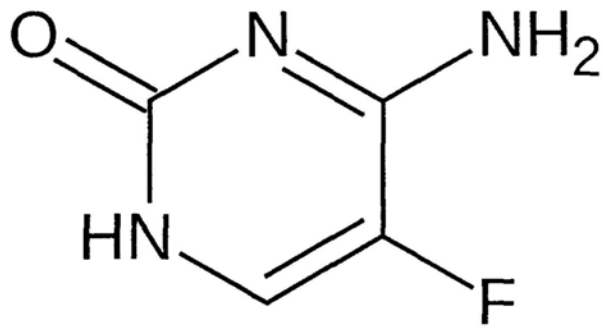


Fig. 1.9 Stucture of flucytosine

1.2.5 Others

Other promising reagents in preclinical development include inhibitors of: sphingolipids biosynthesis; multidrug efflux; protein synthesis (such as the sordarins); lipid modification (such as the myristoyl-N-transferase); and energy conversion at the cell surface (Monk and Cannon, 2002).

1.3 Antifungal drug resistance

The opportunistic human pathogens of *C. albicans* and other non-*albicans* species have recently acquired considerable significance not only from the mortality of their infections, but also from their resistance against antifungal agents, such as the multidrug resistance of azoles after widespread and prolonged use. The antifungal resistance is not restricted to *C. albicans*. The non-*albicans* species, including *C. parapsilosis*, *C. krusei*, *C. glabrata* and *C. tropicalis* also demonstrated this characteristic (Prasad and Kapoor, 2005; Bennett *et al.*, 2004; Coleman *et al.*, 1998). Both *C. albicans* and non-*albicans* species have a variety of antifungal resistance mechanisms (Table 1.1).

Table 1.1 Drug resistance mechanisms by *Candida* species

Candida species	Antifungal agents	Target	Resistance mechanisms	Reference
C. albicans	Azoles	Cytochrome P450 14α-demethylase	Point mutation in the target enzyme to alter the affinity of the enzyme to azoles	Lamb *et al.*, 1997; Kelly *et al.*, 1997
		ABC transporters	Overexpression of ABC multidrug transporters	White, 1997
	Amphotericin B	Membrane ergosterol	Increase membrane fluidity	Cuenca-Estralla *et al.*, 2001a; Kelly *et al.*, 1997
	Nystatin		Alter the membrane sterols	Martin and Dinsdale, 1982
C. krusei	Azoles	Cytochrome P450 14α−demethylase	Reduce the susceptibility of target enzyme to azoles	Venkateswarlu *et al.*, 1996
		ABC transporters	Overexpression of the target transporters and reduce the intracellular drug accumulation	Katiyar and Edlind, 2001
	Amphotericin B	Membrane ergosterol	Decrease membrane ergosterol content	Safe *et al.*, 1977
	Nystatin		Membrane ergosterol absence	Mas and Pina, 1980
C. glabrata	Azoles	ABC transporters	Overexpression of the transporters and mitochondrial loss	Redding *et al.*, 2003; Sanglard *et al.*, 2001;
	Amphotericin B	Membrane ergosterol	Decrease membrane ergosterol content	Sterling *et al.*, 1996
	Nystatin		Membrane ergosterol absence	Krcmery and Barnes, 2002
C. tropicalis	Azoles	ABC transporters	Overexpression of the efflux pumps	Barchiesi *et al.*, 2000; Jandourek *et al.*, 1999
	Amphotericin B	Membrane ergosterol	Decrease membrane ergosterol content	Krcmery and Barnes, 2002

1.4 Discovery of novel antifungal agents

Fungi have played an increasingly important role in the clinical settings. However, unlike the wide range of antibiotics available to treat bacterial infections, there are very few antifungal agents available for the treatment of systemic fungal infections and the first-line antifungal drugs also have a variety of limitations. So there is a great need to search for novel compounds with higher effectiveness and security (Kirsch and DiDomenico, 1994). The antifungal agents used over the last 30 years are mainly derived from a few structural classes. Rather than seeking novel classes of antifungal agents, pharmaceutical companies preferred to improve the approved drugs to give better therapeutic properties such as increasing potency and selectivity, or enhancing drug delivery and bioavailability (Walsh *et al.*, 2000).

1.4.1 Traditional screening

The classical method to find antifungal agent was by looking at its inhibitory ability of fungal growth on agar plate or in the tube. This method was based on the agents' ability to inhibit the fungal growth when diffusing through an agar solidified medium or incubating in the liquid medium. However, the researchers encountered a big problem of their repetitive discovery of known compounds. Screening of the compound libraries and natural products has led to the discovery of most of the current antifungal agents and it is still a primary method to search for novel lead compounds with activity against unknown or unanticipated targets (Silver and Bostian, 1990)

1.4.2 Mechanism-based screening

This screening method is based on the action of antifungals against selected enzymatic or molecular targets, which avoids the shortcoming of rediscovery in the traditional method. This method is particularly valuable to search for the novel antifungal agents because fungal cells are eukaryotes and most agents that could inhibit the fungal growth are also active against mammalian cells, which decreases the detection of toxins and avoids the subsequent laborious characterization of a lot of substances which may be therapeutically meaningless. More importantly, this screening method provides an advantage with high level of sensitivity, resulting in the discovery of novel agents which might escape from the traditional screening (Sykes *et al.* 1981).

1.4.3 Medicinal chemistry

The use of chemical synthesis can provide a variety of pharmaceutical compounds. This method can proceed without knowing the target from theoretical targets based on existing endogenous ligands. This approach has been successful in the discovery of novel agents in renal, cardiovascular and central nervous system areas and also valuable in the antifungal area. The triazoles and imidazoles are synthetic compounds and comprise one of the clinically used antifungal groups (Saag and Dismukes, 1988).

1.5 Research background

Compound library is one source of antimicrobial agents among today's world of chemical libraries and combinatorial syntheses, but they offer an almost unlimited reservoir of unique structures. Also, they avoid the tedious and intensive labor of the isolation, purification and identification of novel anti-Candidal compounds from plants. Coupled with complete genome sequences of target and surrogate organisms, antimicrobial drug discovery from compound library may be a plausible alternative (Ernst and Rogers, 2005). In the present study, a compound library with 400 members was screened for its antifungal activity against a panel of 20 *Candida* strains. The results showed that metergoline, purpurin and baicalein were three novel antifungal agents worth detail investigation.

1.6 Structure-activity relationship

The considerable biological activities of flavones including baicalein have recently been reviewed (Min, 2009). Furthermore, the chemistry of these structures is very interesting because there are several free hydroxyl groups worth to be modified. Combinatorial chemistry has been a promising tool in drug discovery. Potential compounds possessing functional groups are derivatized by chemical modifications (e.g. acylation, glycosylation, oxidation and reduction). Some reports have shown that O-acylated or N-acylated derivatives had more potent antimicrobial activities, comparing with their precursors (Dogan *et al.*, 1998; Wakabayashi *et al.*, 1999; Hu *et al.*, 2007). In order to acquire more potent compounds and investigate the role of

hydroxyl group of baicalein in the antifungal activity, we chemically synthesized four compounds of acylated baicalein and measured their antifungal activities.

Chapter 2

Materials and Methods

2.1 Organisms, cell line and culture conditions

Candida ATCC strains were obtained from the American Type Culture Collection and other *Candida* clinical isolates were obtained from the archival collection of Oral BioSciences, Faculty of Dentistry, The University of Hong Kong. They were routinely cultured onto Sabouraud's dextrose agar or YM agar at 30 °C. The human HepG2 and WRL68 cell lines were obtained from the American Type Culture Collection. They were maintained in RPMI 1640 medium supplemented with 10% FBS and a penicillin-streptomycin mixture at 37 °C in a humidified atmosphere containing 5% CO_2 in air.

2.2 Antifungal agents

Metergoline, purpurin and baicalein were purchased from TimTec Inc. (Newark, DE, USA) and the purity was more than 99%. Buspirone, ketanserin, cyproheptadine, clozapine, cisapride and ondansetron were purchased from Sigma-Aldrich (St. Louis, MO, USA). AMB and FLC were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Pfizer Inc. (New York, NY, USA) respectively. Baicalein derivatives were synthesized in the Department of Chemistry, The Chinese University of Hong Kong. All agents were dissolved in distilled dimethylsulfoxide (DMSO) and stored at -20 °C until use. The DMSO concentration was 1% in all assays.

2.3 Minimal inhibitory concentration assay

Minimal inhibitory concentration (MIC) was determined by a broth microdilution assay in accordance with the CLSI guidelines. Briefly, fungal cell suspension was incubated with antifungal agent (1 µl) and RPMI 1640 (99 µl) (Invitrogen, CA, USA). Antifungal agents were tested in a series of concentration in two-fold dilution. After 24 h of incubation at 35 °C in a moist, dark orbital shaker, fungal growth was measured by a spectrophotometric method. The flat-bottom 96-well microtiter plates were shaken for 2 min to obtain a uniform suspension prior to spectrophotometric measurement at 595 nm. The MIC was defined as the lowest concentration of the compound at which there was 80% inhibition of visible growth, as indicated by the absorbance values, when compared with the solvent control (1% DMSO) (Cuenca-Estrella *et al.*, 2001b; Kustimur *et al.*, 2003).

2.4 Minimal fungicidal concentration assay

Minimal fungicidal concentration (MFC) was determined as described previously (Lass-Flörl *et al.*, 2003). Briefly, 100 µl content from each well showing complete growth inhibition and from growth control wells was spread onto YM agar and colony-forming unit was counted after 24 h of incubation at 35 °C. The MFC was defined as the lowest concentration of the compound that resulted in 99% killing of the initial incubating cell number.

2.5 Cell viability assay

The proliferation of compound-treated fungal cells was assessed using a tetrazolium-based viability assay (Promega, Madison, WI) (Shirtliff *et al.*, 2009) (Fig. 2.1). 1×10^7 cells/ml fungal cell suspension (100 µl) in RPMI 1640 (Invitrogen, CA, USA) was treated by compound at different concentrations in the wells of 96-well microtiter plates. The plates were incubated for 24 h at 35 °C with shaking. Following incubation, 100 µl of the XTT reagent was added to each well and the plates were incubated at 37 °C for 4 h. Then 100 µl of each sample was transferred to a fresh plate and the colorimetric change at 490 nm was measured with a microplate reader (BIO-RAD, model 3550).

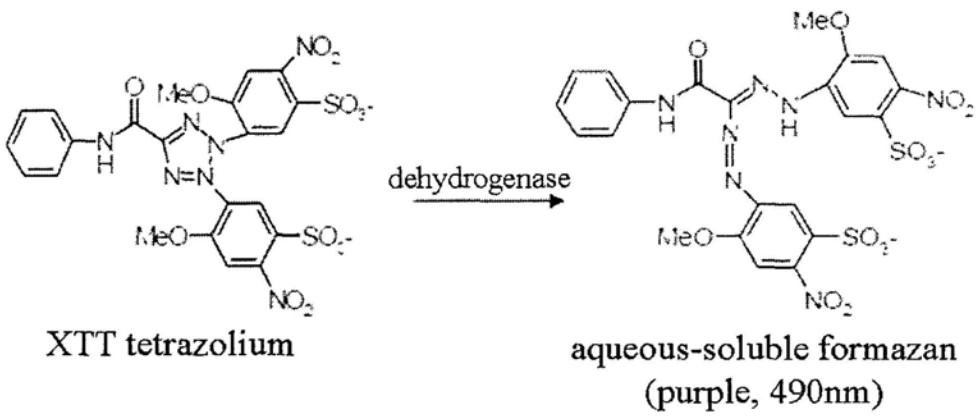


Fig. 2.1 Principle of the XTT assay in measuring cell viability.

2.6 Cytotoxic effects on mammalian cells

The cytotoxic effects of compound were measured by a MTT method (Cui *et al.*, 2008). Aliquots (100 µl) of 3×10^5 cells/ml of HepG2 and WRL68 cells were seeded in 96 well flat-bottomed plates and they were incubated at 37 °C overnight. The

incubation medium was replaced with compound at different concentrations over 48 h. The ability of the drug to inhibit cell growth was determined by adding 100 µl MTT reagent and incubated for another 4 h. After removing the supernatant, 150 µl DMSO was added to dissolve the formazan. The OD value was measured at 570 nm.

2.7 Determination of post-antifungal effect (PAFE)

PAFE of antifungal agents were examined in accordance with a well-known method (Anil *et al.*, 2000). Briefly, fungal cell suspension (5×10^6 cells/ml) was incubated with different concentrations of antifungal agent (1 ×, 2 × or 4 × MIC) at 35 °C for 1 h. Antifungal agents were removed by washing the cells thrice. Cells were adjusted to 5×10^3 cell/ml and 100 µl was added into a 96-well plate. Plates were placed in a shaker and incubated at 35 °C for 48 h. At different time intervals, growth of fungal cells was monitored by a plate reader at 595 nm. The duration of PAFE was calculated by using the formula PAFE = $T - C$, where T and C are the time periods required for the relative optical density of the drug-exposed cell suspension and the drug-free control cell suspension, respectively, to reach the same absorbance level at 0.05.

2.8 Combination study

Interactions between compounds tested and AMB or FLC were assessed by a checkerboard assay in microdilution. Fungal cell suspension was incubated with

different concentrations of compound tested in the presence of either FLC (0.125 μg/ml to 64 μg/ml) or AMB (0.125 μg/ml to 16 μg/ml) in two-fold dilution. The fractional inhibitory concentration index (FICI) was defined as the sum of the MIC of each compound when used in combination divided by the MIC of the compound alone. The interactions were categorized as either synergistic (FICI ≤0.5), indifferent (0.5< FICI ≤4) or antagonistic (FICI >4) (Oliveira *et al.*, 2005).

2.9 Rhodamine 6G (R6G) efflux assay

R6G assay was used to investigate the effect of compound tested on the cell permeability on *Candida* species (Holmes *et al.*, 2008). Fungal cell suspension (5×10^7 cells/ml) was starved for 2 h, followed by addition of R6G to a final concentration of 10 μM. Cell suspension was incubated at 35 °C with shaking (200 rpm) for 1 h to allow R6G accumulation. Fungal cells were then washed thrice with phosphate buffered saline (PBS) and the cell concentration was adjusted to 5×10^7 cells/ml. Glucose (1 mM) and different concentrations of compounds were added and incubated for a further 2 h. Cells were removed by centrifugation, and 100 μl of the cell supernatant was transferred to 96-well flat-bottom microplates. Intensity of R6G fluorescence was determined by fluorescence spectrophotometer with excitation wavelength of 485 nm and emission wavelength of 535 nm.

2.10 Effect on extracellular phospholipase production

The effect of compound on phospholipase production in *Candida* species was determined by using the egg yolk agar method as described previously (Samaranayake *et al.*, 1984). Egg yolk agar contained 13 g Sabouraud's dextrose agar, 11.7 g NaCl, 0.11 g $CaCl_2$ and 10% sterile egg yolk emulsion in a final volume of 100 ml (Oxoid, UK). Fungal cells were first treated with compound at different concentrations for 2 h. A 5-µl aliquot (10^7 cells/ml) was spread on the surface of egg yolk agar, air dried at room temperature and incubated at 37 °C for 72 h. Diameters of the fungal colony and the precipitation zone around the colony were measured. Extracellular phospholipase production (Pz) was determined as the ratio of the diameter of the colony to the total diameter of the colony and the precipitation zone (Fig. 2.2). A Pz value equals to 1.00 indicates that there is no phospholipase production while a Pz value ≤0.63 indicates high level of phospholipase production (Price *et al.*, 1982).

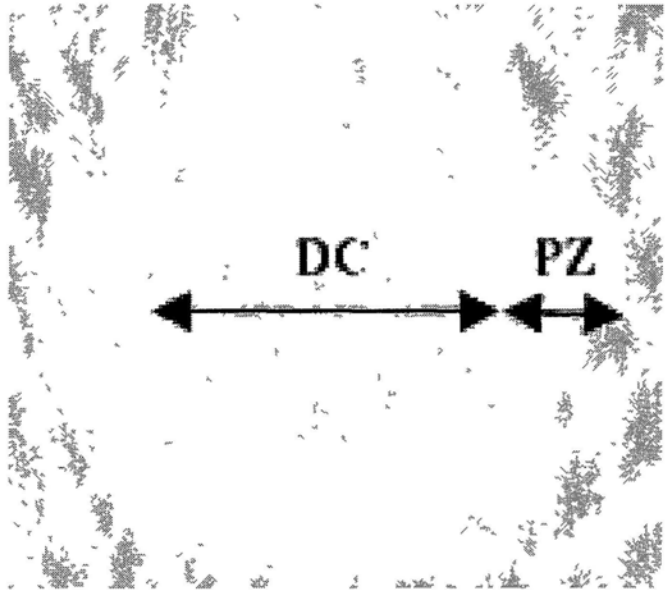


Fig. 2.2 Egg yolk assay to evaluate phospholipase production. DC: diameter of the yeast colony; PZ: precipitation zone (Kadir *et al.*, 2007).

2.11 Reactive oxygen species (ROS) accumulation

The effect of compound tested on intracellular ROS level in *Candida* species was analyzed as described (Kobayashi *et al.*, 2002). Briefly, cell suspension was adjusted to 5×10^6 cells/ml and treated with compound at different concentrations in the presence or absence of ascorbic acid or mannitol at 35 °C for 3 h. After removal of compounds by washing in PBS, fungal cells (100 µl) were transferred into 96-well flat-bottomed microtiter plates and 2',7'-dichlorofluorescin diacetate (DCFDA) (Molecular Probes, CA) was added to a final concentration of 20 µM and incubated at 37 °C for 1 h. The intensity of fluorescence was measured in a fluorescence plate reader (TECAN Polarion, Tecan UK Ltd, Theale, UK) with 485 nm excitation and 535 nm emission (Fig. 2.3).

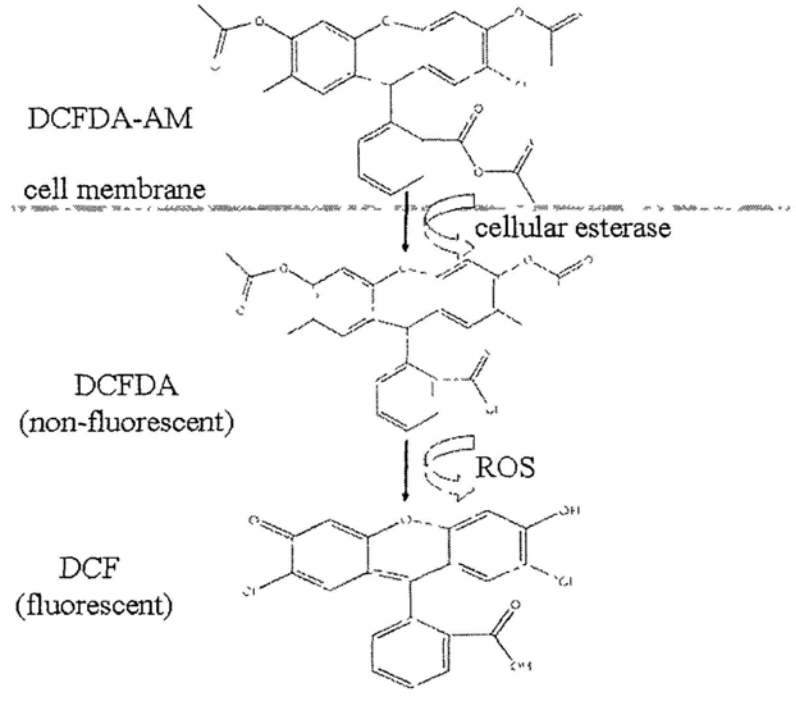


Fig. 2.3 Principle of the DCFDA assay in measuring ROS level.

2.12 Mitochondria depolarization

The effect of compound on the mitochondrial membrane potential (MMP) of *Candida* species was analyzed as described (Pina-Vaz *et al.*, 2001). Briefly, cell suspension was adjusted to 5×10^6 cells/ml and treated with compounds at different concentrations at 35 °C for 2 h. The cells were washed thrice, resuspended in PBS at 10^6 cells/ml and incubated with 0.25 μM 5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethyl-imidacarbocyanine iodide (JC-1) (Molecular Probes, CA) at 35 °C for 15 min. The fluorescence intensities at FL-1 (green fluorescence, 525 nm) and FL-2 (red fluorescence, 595 nm) were recorded by flow cytometry (Beckman Coulter, Inc., CA) and the results were expressed as a ratio of the mean values at FL-2 and FL-1 (Fig. 2.4).

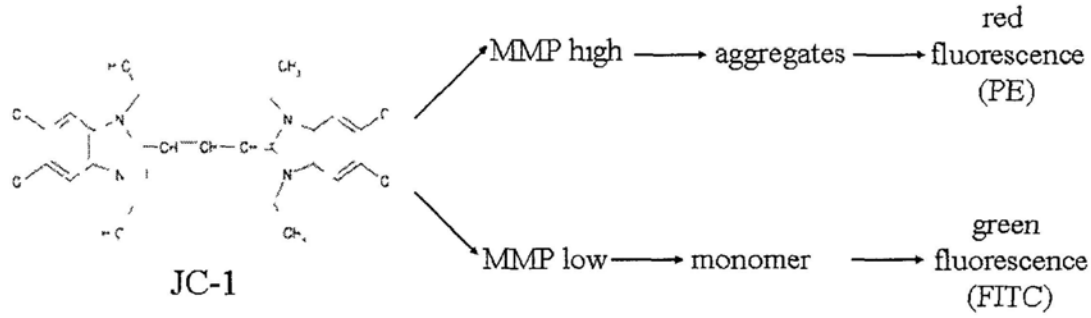


Fig. 2.4 Principle of the JC-1 assay in measuring the mitochondrial membrane potential.

2.13 Annexin V/PI staining

Apoptosis was tested by annexin V/PI staining (Andrés *et al.*, 2008; Dai *et al.*, 2009). Cell suspension was adjusted to 5×10^6 cells/ml and treated with compounds at

different concentrations at 35 °C for 12 h. After removal of compound by washing in PBS, the cells (10^7 cells/ml) were suspended in sorbitol buffer (1.2 M sorbitol, 0.5 mM $MgCl_2$, 35mM K_2HPO_4, 30 mM 2-mercaptoethanol [pH 6.8]) and treated with lyticase (50 U/ml) and glusulase (5.5%) in the same buffer (1h, 35 °C) to digest the cell wall. The protoplasts were washed in annexin binding buffer (10 mM HEPES [pH 7.4], 40 mM NaCl, 50 mM $CaCl_2$, 1.2 M sorbitol), and annexin V binding assays were performed in the same buffer by the addition of 20 µl of annexin V/ml and 20 µg/ml of PI, incubating for 10 min in the dark. Signals were immediately detected by flow cytometry (Beckman Coulter, Inc., CA). The cell population of interest was gated on the basis of the forward-scatter and side-scatter properties. The different labelling patterns in the bivariate PI/annexin V analysis identify the different cell populations: FITC negative and PI negative were designated as viable cells; FITC positive and PI negative as apoptotic cells (cells with PS externalization); and FITC positive and PI positive as late apoptotic or necrotic cells.

2.14 TUNEL assay

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay was performed to confirm the occurrence of apoptosis in *Candida* species after compound treatment by using the In Situ Cell Death Detection Kit (Fluorescein) (Roche, Penzbery, Germary) (Shirtliff *et al.*, 2009) (Fig. 2.5). Briefly, after 12-h incubation with different concentrations of compounds, fungal cells were washed thrice with PBS and fixed with 2% paraformaldehyde at 20 °C for 1 h. The fungal cells were rinsed thrice with PBS and then incubated with permeabilization solution

(0.1% Triton X-100, 0.1% sodium citrate) on ice for 2 min. The fungal cells were rinsed twice in PBS and labeled with 50 µl TUNEL reaction mixture at 37 °C for 1 h in a humidified incubator in the dark. The fluorescence was quantified by using FACSort™ flow cytometer at 530 nm. Negative (no enzyme added) control was included for comparison and the data were analyzed with the WinMDI 2 9 software.

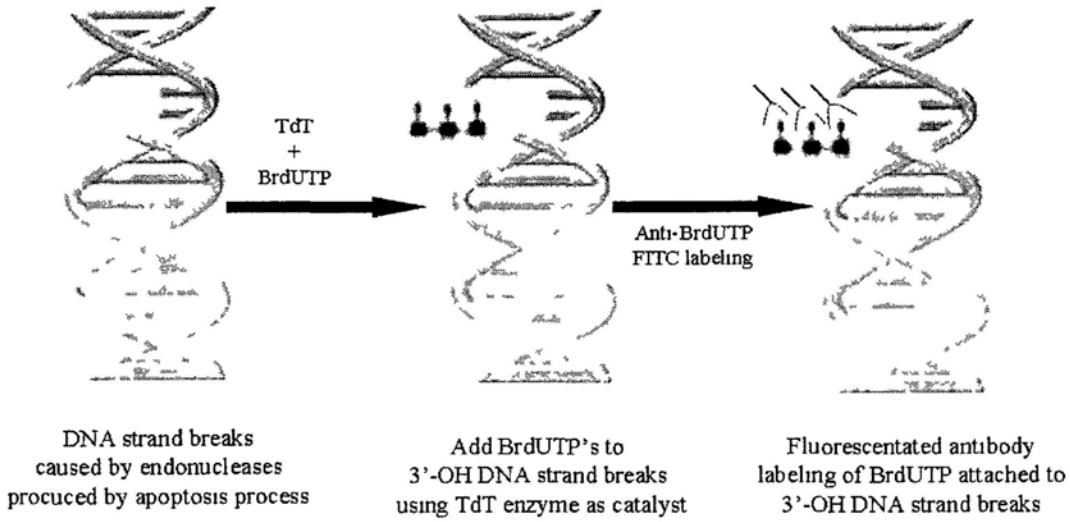


Fig. 2.5 Principle of the BrdU TUNEL assay in measuring DNA fragmentation.

2.15 Synthesis of baicalein derivatives

All the reactions were performed under an atmosphere of nitrogen. Pyridine was distilled from calcium hydride. All other solvents and reagents were of reagent grade and used as received. ^{1}H and $^{13}C\{^{1}H\}$ NMR spectra were recorded on a Bruker Advance III 400 spectrometer (^{1}H, 400; ^{13}C, 100.6 MHz) in deuterated solutions. Spectra were referenced internally using the residual solvent (^{1}H: 7.26 for $CDCl_3$; ^{13}C: 77.0 for $CDCl_3$) resonances relative to $SiMe_4$.

Baicalein and anhydride were added into a 25 ml flask with 1.5 ml pyridine. The reaction mixture was stirred and refluxed for 20 h. Then 20 ml of water was poured into the flask and the flask was kept in an ice-bath until the solid precipitated. The suspension was filtered and purified by column chromatography on silica gel using chloroform as eluent. All new compounds were identified by ^{3}H-NMR and ^{13}C-NMR (Fig. 2.6).

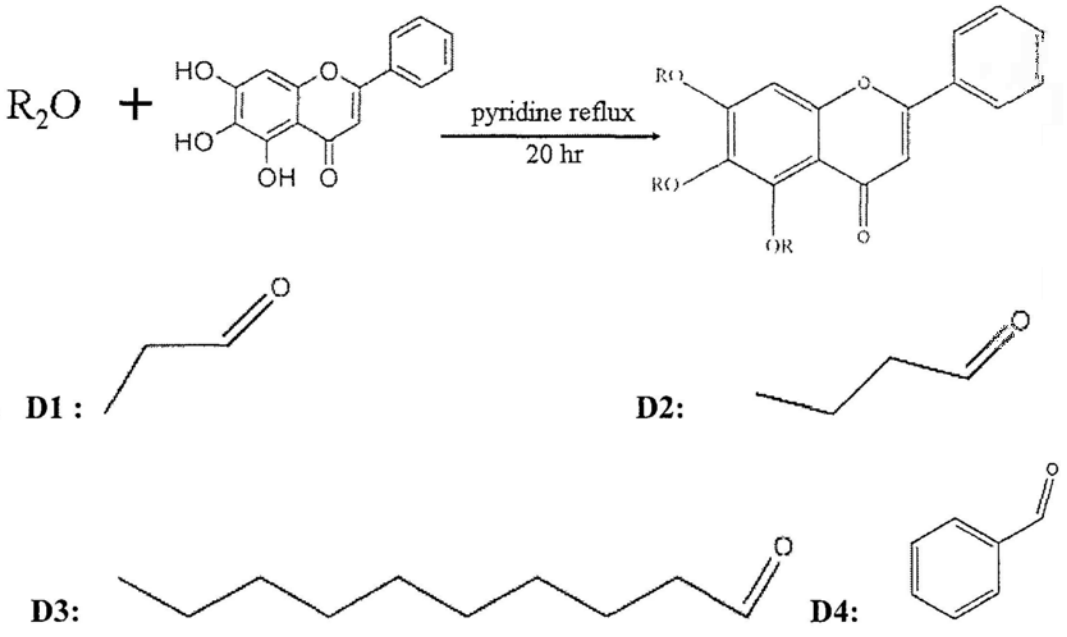


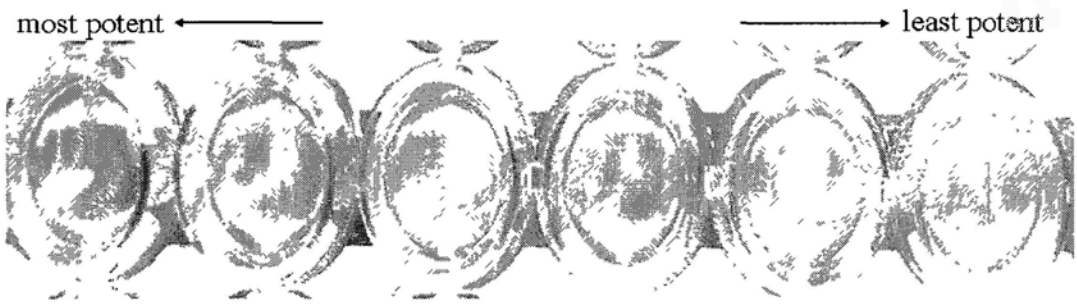
Fig 2.6 Synthetic route to compounds D1-D4.

2.16 Statistical analysis

All experiments were performed in triplicate on three separate occasions. All data were expressed as mean values with the corresponding standard deviations (SD). Student's *t* test was employed to assess the statistical significance of treated versus untreated samples. A p-value < 0.05 was considered statistically significant.

Chapter 3
Compound Library Screening

A compound library with 400 members derived from plants was obtained from TimTec Inc. Its antifungal activity against a panel of 20 *Candida* strains was studied. The final concentration of compounds was 20 μM. After incubating with compounds in 96 well-plate for 24 h, fungal cell suspension was examined by naked eyes on a piece of paper imprinted with black lines. Comparing with the control, the results can be categorized into five levels (Fig. 3.1). The smaller the number, the clearer is the well, which means the more potent the compound in its antifungal activity.



0	optically clear
1	slightly hazy
2	prominent reduction in turbidity compared with that of negative control
3	slight reduction in turbidity compared with that of negative control
4	no reduction in turbidity compared with that of negative control

Fig. 3.1 Evaluation and scoring of the result.

According to the screening results (see appendix Ⅰ), we screened out 14 compounds and they were divided into two groups. The first group is No. 93, 178, 205, 225, 269, with the average scale less than or equal to 1, which means they have potent

antifungal activities. The second group is No. 51, 100, 214, 233, 247, 330, 357, 378, 379, with the average scale about 2 and having moderate effect. The remaining others are regarded as having no antifungal effect.

Among the first group, we find out that No.225 is anisomycin, a known compound with antifungal effect, which can interfere with protein synthesis by inhibiting peptidyl transferase. No. 269 is cycloheximide, widely used in biomedical research as a protein synthesis inhibitor *in vitro* in eukaryotic cells studies. These two known compounds confirm that our screening method does work. As for the other three compounds in the first group, No. 93 is an anthraquinone, known as purpurin. No. 205 is a flavone, known as baicalein. No. 178 is metergoline.

Metergoline is an ergot derivative (Fig. 3.2). It is not only a dopamine agonist with actions and uses similar to bromocriptine, but it is also a serotonin receptor antagonist (Miller *et al.*, 1992; Petrus, 1997). It has been used in disorders associated with hyperrolactinaemia and also used to inhibit lactation, to treat gastrointestinal disorders, prophylaxis of migraine and other vascular headaches. Recently, serotonin and its reuptake inhibitors have been suggested as potential novel antifungal candidates due to their efficacy against *Candida* and *Aspergillus* species (Lass-Flörl *et al.*, 2001; Mayr *et al.*, 2005; Lass-Flörl *et al.*, 2003a). Therefore, in the present study we decided to study the antifungal activity of metergoline against FLC resistant *C. krusei* isolates and investigated its antifungal mechanisms.

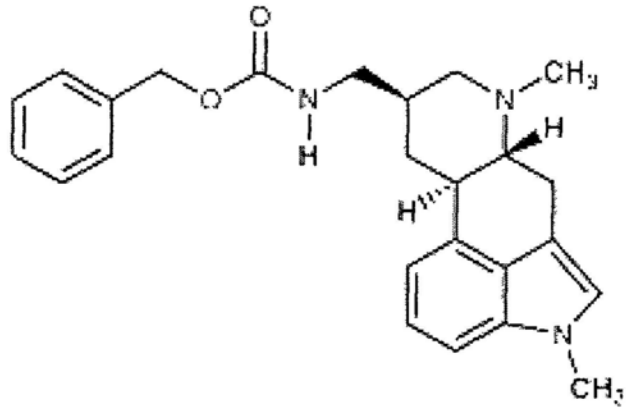


Fig. 3.2 Structure of metergoline

Purpurin, 1,2,4-trihydroxy-9,10-anthraquinone (Fig. 3.3), together with alizarin, can be found in the roots of the madder plant. It is a natural anthraquinone pigment currently used as food colouring agent and for cotton printing. Anthraquinone pigments have been traditionally used for textile staining, color paints, food coloring and medicinal treatments. Anthraquinone including purpurin and alizarin has been reported to show suppressive effects on mutagenesis on the activity of individual human cytochrome P450s (Takahashi *et al.*, 2002; Takahashi *et al.*, 2001; Marczylo *et al.*, 1999). Recently, a number of reports have shown the antimalarial, antibacterial and antifungal activities of anthraquinones. In particular, emodin, alizarin, chrysophanol and rhein exhibited antifungal activity against *C. albicans* (Agarwal *et al.*, 2000; Kong *et al.*, 2009; Xiang *et al.*, 2008). However, virtually nothing is known about the antifungal activities of purpurin on *Candida* species.

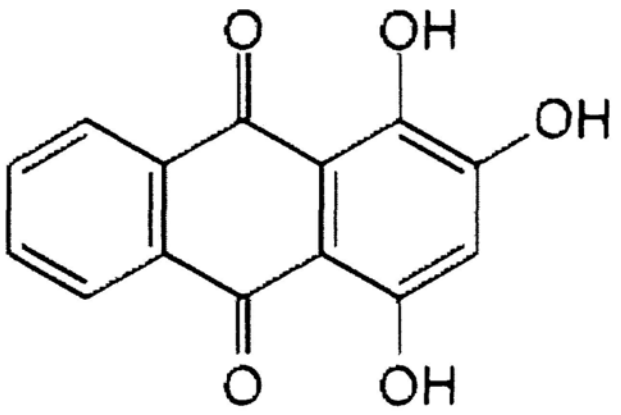


Fig. 3.3 Structure of purpurin

Baicalein, 5,6,7-trihydroxyflavone (Fig. 3.4), is a flavone originally isolated from the roots of *Scutellaria baicalensis*. Baicalein is an inhibitor of CYP2C9, an enzyme of the cytochrome P450 system that metabolizes drugs in body (Si *et al.*, 2009). Baicalein has been found to have antioxidant, neuroprotective, antibacterial and antiviral activities (Lin *et al.*, 2007; Chang *et al.*, 2007a; Chang *et al.*, 2007b; Wu *et al.*, 2001). Some recent studies showed that baicalein also exhibited antifungal activity. It had antimicrobial activity and synergistic effect with other antibiotics (Huang *et al.*, 2008) and also possessed antifungal activity against *C. albicans* biofilms and induced programmed cell death in *C. albicans* (Cao *et al.*, 2008; Dai *et al.*, 2009). However, the antifungal activity of baicalein against FLC-resistant *C. krusei* has never been reported, so study was carried out to highlight the extension of antifungal spectrum of baicalein.

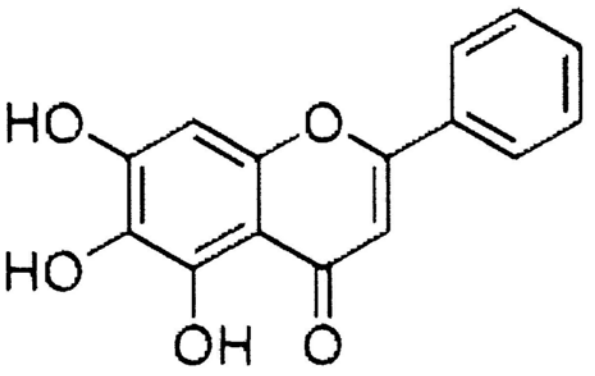


Fig. 3.4 Structure of baicalein

To the best of our knowledge, *in vitro* antifungal activity of purpuirn and metergoline has not been previously reported. Baicalein was reported with antifungal activity against *C. albicans* (Cao *et al.*, 2008; Dai *et al.*, 2009), however, its antifungal activity against *C. krusei* has never been reported. So we proceeded to examine the effect of these three compounds and investigated their antifungal mechanisms.

Chapter 4

Antifungal Activities of Metergoline against *Candida krusei*

4.1 Introduction

There are nearly 150 yeast species presently classified in the genus *Candida*. Among these, *C. albicans*, *C. tropicalis* and *C. glabrata* comprise > 80% of clinical *Candida* isolates (Samaranayake and Samaranayake, 1994). However, extensive use of antifungal agents and rise in compromised host population such as AIDS patients have increased the non-*albicans* species related infections, particularly those of *C. krusei*, *C. tropicalis* and *C. glabrata* (Pelletier *et al.*, 2005; Krcmery and Barnes, 2002; Drago *et al.*, 2004). In particular, *C. krusei* related infections have dramatically increased because of its intrinsic resistance to FLC and decreased susceptibility to AMB and become one of the most frequent emerging pathogens, especially among patients with acute leukemia (Abbas *et al.*, 2000; Samaranayake, 1997; Muñoz *et al.*, 2005; Pfaller *et al.*, 2008). Alarmingly, failure of the novel antifungal agent caspofungin (a class of echinocandins) against *C. krusei* infections has also been reported (Mora-Duarte *et al.*, 2002; Pelletier *et al.*, 2005).

Conventionally, both superficial and deep-seated forms of candidiasis are treated with antifungal agents such as polyenes, azoles and echinocandins (Arikan *et al.*, 2007; Petrikkos and Skiada, 2007; Prasad and Kapoor, 2005). Although treatments by conventional antifungal agents seem promising, toxic side effects on liver, poor gastrointestinal absorption and emergence of resistant clinical *Candida* strains have been documented (Muñoz *et al.*, 1991).

The clinical significance and the severity of drug resistance in candidiasis pose an urgent need to search for antifungal agents with novel mechanisms of action. After screening a compound library, we found that metergoline, a serotonin receptor antagonist, exhibited potent antifungal activity, especially against FLC resistant *C. krusei* isolates. A recent study on sertraline has shown that inhibition of phospholipase relieved apoptosis in *Leishmania donovani* (Palit and Ali, 2008). It is thus hypothesized that the action mechanisms of metergoline in *C. krusei* may be related to the initiation of apoptosis. Many stress stimuli, such as ROS, chemotherapeutic drugs, can induce mitochondrial apoptosis and the understanding of the mitochondrial apoptotic pathway (Fig. 4.1) is important for effective chemotherapy (Asakura and Ohkawa, 2004). Here we studied the outcomes of cell after being treated with metergoline by investigating several classical apoptotic markers. These included elevation of intracellular level of ROS, depolarization of MMP, loss of membrane asymmetry and DNA fragmentation. These biochemical events confirmed that metergoline may trigger apoptotic response in *C. krusei*.

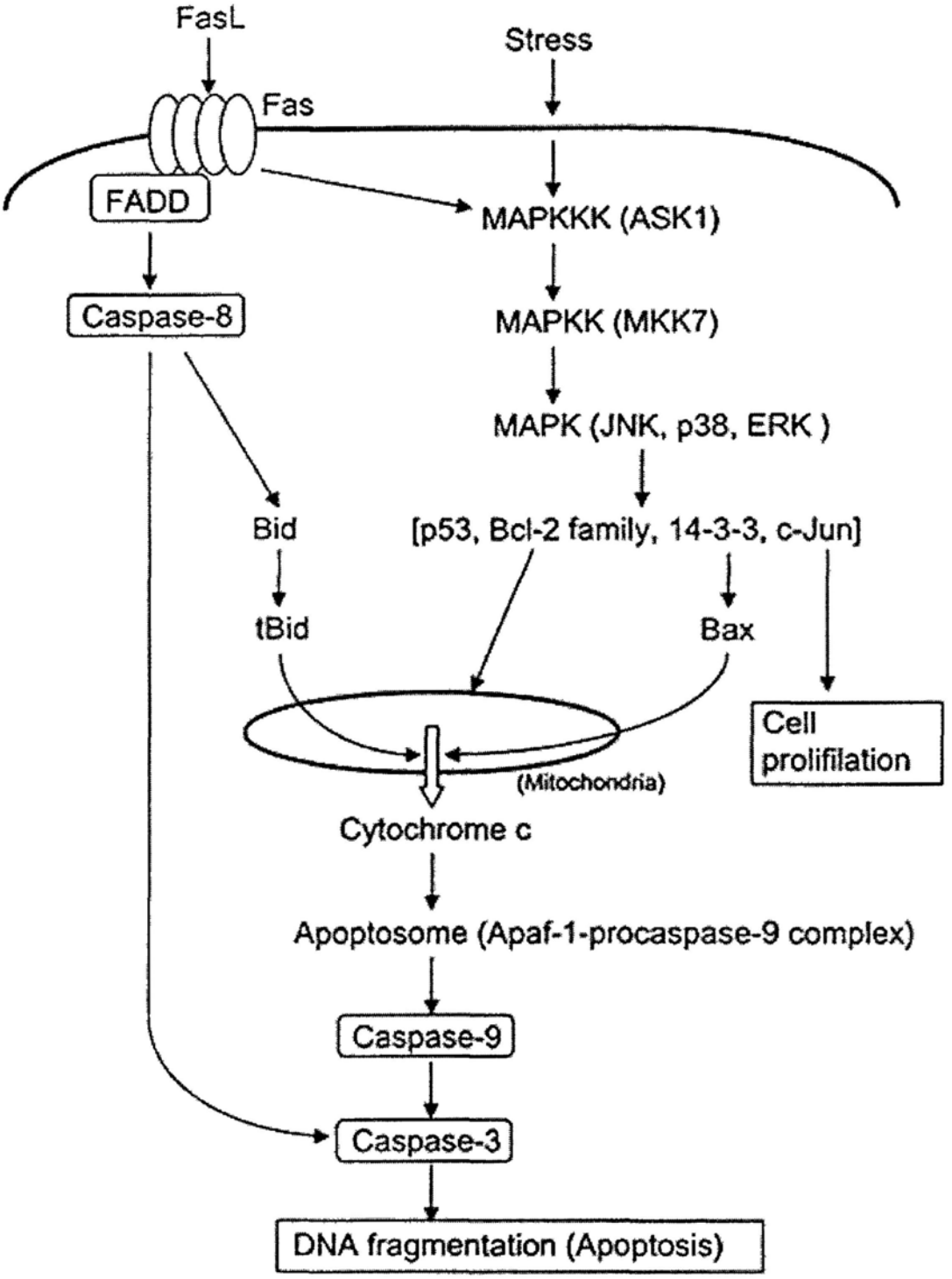


Fig. 4.1 Apoptosis signal transduction (Asakura and Ohkawa, 2004).

4.2 Results

4.2.1 MIC, MFC, synergistic effect between metergoline and AMB, FLC

A summary of MIC and MFC values of metergoline is shown in Table 4.1. Metergoline alone exhibited a potent antifungal activity against *C. krusei* after 24 h of incubation and the MIC values were the same at 4 μg/ml (10 μM). Metergoline was denoted as fungicidal agent with the MFC values of 8 μg/ml (20 μM). Combination study demonstrated that metergoline exerted potent synergism in combination with AMB at subinhibitory concentrations against the fourteen *C. krusei* isolates. Other six serotonin receptor antagonists showed weaker antifungal activity on *C. krusei* and the MIC were all higher than 40 μM (Table 4.2).

Table 4.1 The MIC and MFC values of metergoline, AMB, FLC and the *in vitro* combinational activity against *C. krusei* isolates after 24 h of incubation.

C. krusei isolates	Metergoline (μg/ml)		AMB (μg/ml)		FLC (μg/ml)		FICI*	
	MIC	MFC	MIC	MFC	MIC	MFC	Metergoline + AMB	Metergoline + FLC
ATCC 6258	4	8	1.00	2.0	32	>128	0.375[a]; (0.5/0.25)[c]	0.625[b]; (0.5/16)[c]
CK1	4	8	0.25	1.0	64	>128	0.500[a]; (1/0.06)[c]	0.750[b]; (1/32)[c]
CK2	4	8	0.25	1.0	64	>128	0.375[a]; (0.5/0.06)[c]	0.500[a]; (1/16)[c]
CK3	4	8	1.00	2.0	32	>128	0.375[a]; (1/0.125)[c]	0.625[b]; (0.5/16)[c]
CK4	4	8	0.25	0.5	64	>128	0.500[a]; (1/0.06)[c]	0.375[a]; (0.5/16)[c]
CK5	4	8	0.25	1.0	32	>128	0.500[a]; (1/0.06)[c]	0.625[b]; (0.5/16)[c]
CK9	4	8	1.00	2.0	64	>128	0.375[a]; (1/0.125)[c]	0.375[a]; (0.5/16)[c]
CK13	4	8	0.50	1.0	64	>128	0.500[a]; (1/0.125)[c]	0.500[a]; (1/16)[c]
CK15	4	8	1.00	2.0	32	>128	0.375[a]; (1/0.125)[c]	0.625[b]; (0.5/16)[c]
CK16	4	8	0.50	1.0	64	>128	0.500[a]; (1/0.125)[c]	0.500[a]; (1/16)[c]
CK17	4	8	0.50	2.0	64	>128	0.500[a]; (1/0.125)[c]	0.375[a]; (0.5/16)[c]
CK18	4	8	0.50	1.0	32	>128	0.500[a]; (1/0.125)[c]	0.750[b]; (1/16)[c]
CK20	4	8	0.50	1.0	64	>128	0.375[a]; (1/0.06)[c]	0.500[a]; (1/16)[c]
CK25	4	8	0.50	1.0	64	>128	0.375[a]; (1/0.06)[c]	0.375[a]; (0.5/16)[c]

* FICI= the sum of the MIC of each compound when used in combination divided by the MIC of the compound alone.

[a] Synergistic, [b]Indifferent, [c]MIC values (μg/ml) of each compound in combination

Table 4.2 The antifungal activities of six serotonin receptor antagonists against *C. krusei* isolates after 24 h of incubation.

C. krusei isolates	MIC (μM)					
	Buspirone	Ketanserin	Cyproheptadine	Clozapine	Cisapride	Ondansetron
ATCC 6258	>40	>40	>40	>40	>40	>40
CK25	>40	>40	>40	>40	>40	>40

4.2.2 Antiproliferative effect of metergoline against *C. krusei*

The proliferation of *C. krusei* cells was assayed after treating with different concentrations of metergoline. Results showed that 2 μg/ml metergoline had a little inhibiting effect of 8% and the percentage of inhibitory cells increased in proportion to the metergoline concentration. The percentage reached more than 80% at or above 16 μg/ml (Fig. 4.2).

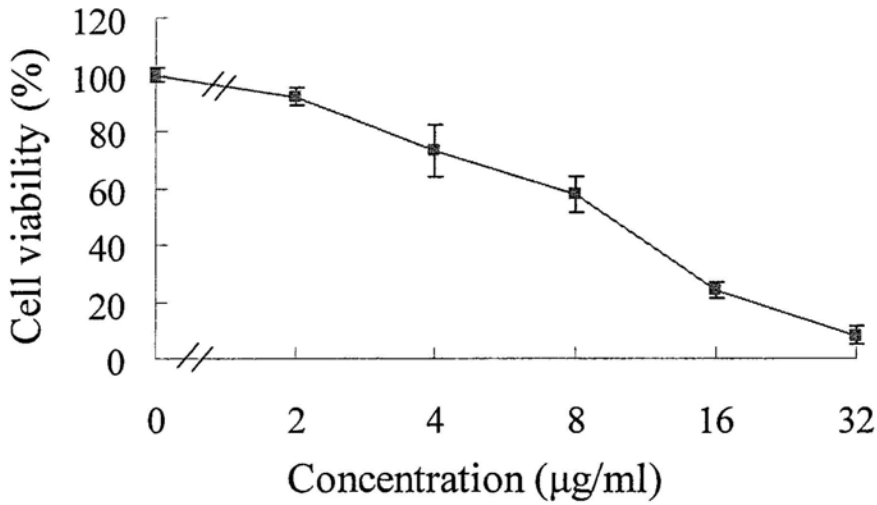


Fig. 4.2 Cell viability of *C. krusei* cells following 24-h treatment with metergoline (0 to 32 μg/ml), as examined by XTT assay. Error bars indicated standard errors of the means (n=3).

4.2.3 Cytotoxic effect of metergoline on mammalian cells

The cytotoxicity of metergoline was tested by performing the MTT assay using human hepatoma cancer cell line (HepG2) and normal liver cell line (WRL68). The effects were represented by IC_{50} values i.e. the concentrations at which 50% of the cells are inhibited from growing. The IC_{50} values of metergoline on HepG2 and WRL68 cell line were similar, both at 8 μg/ml (Fig. 4.3).

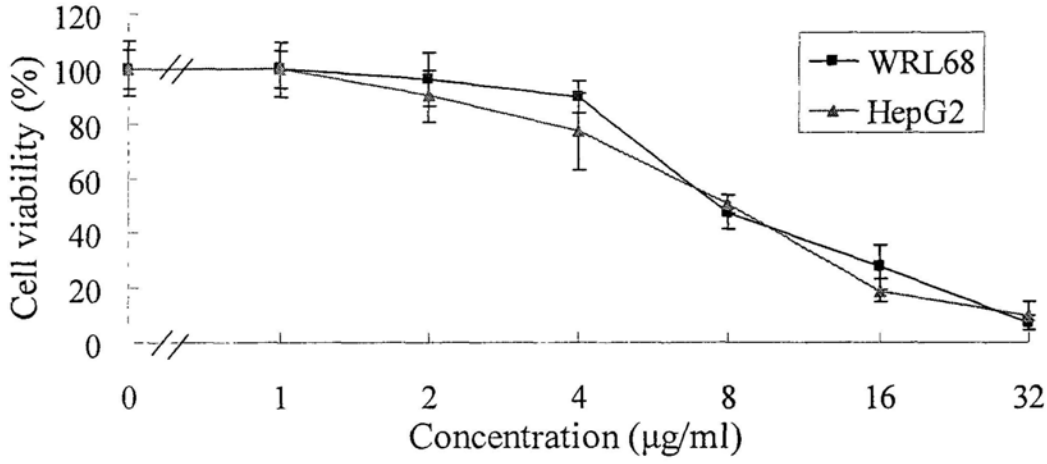


Fig. 4.3 Cytotoxicity of metergoline on mammalian cells following 48-h treatment, as examined by MTT assay. Error bars indicated standard errors of the means (n=3).

4.2.4 PAFE of metergoline on *C. krusei*

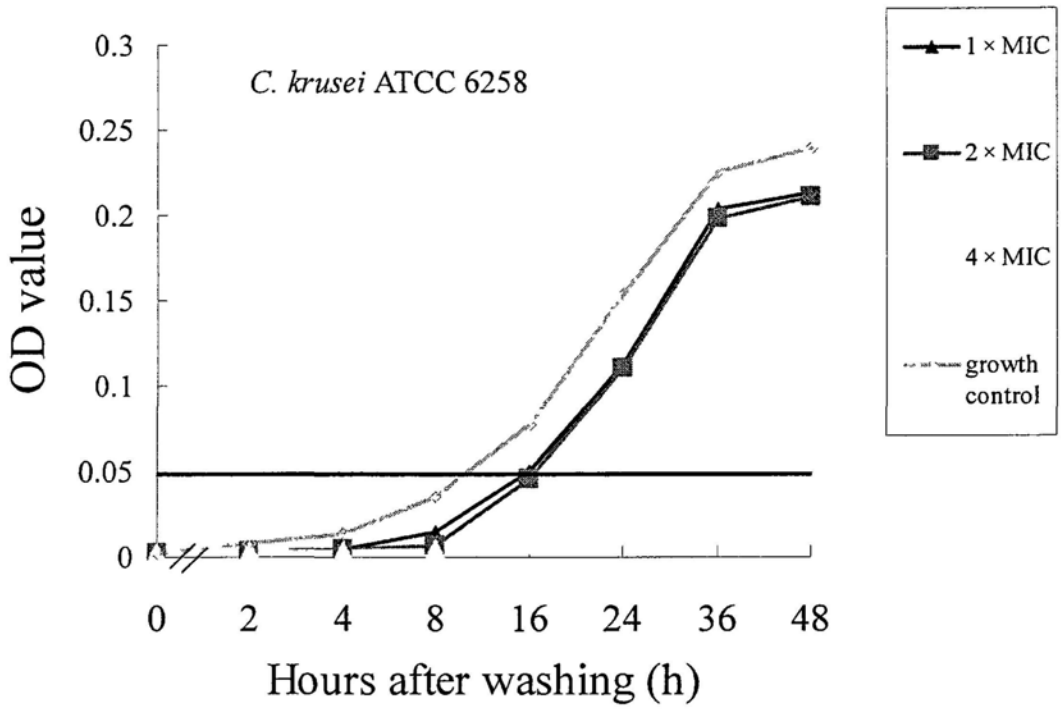
PAFE measures the suppression of fungal growth after limited exposure to antifungal agent and supports clinical relevance for design of dosing regimens, such as the frequency of administration of antifungal agents. In general, PAFE was observed in metergoline-treated *C. krusei* isolates (Fig. 4.4 & Table 4.3).

Table 4.3 PAFE (h)* on *C. krusei* isolates after 1-h exposure to metergoline.

C. krusei isolates	Metergoline		
	1 × MIC	2 × MIC	4 × MIC
ATCC 6258	4.38	7.38	21.00
CK25	3.47	6.90	19.72
Mean	3.93±0.64	7.14±0.34	20.36±0.91

* The value is the average of three independent determinations while the mean refers to the average ± SD of the two *C. krusei* isolates.

A



B

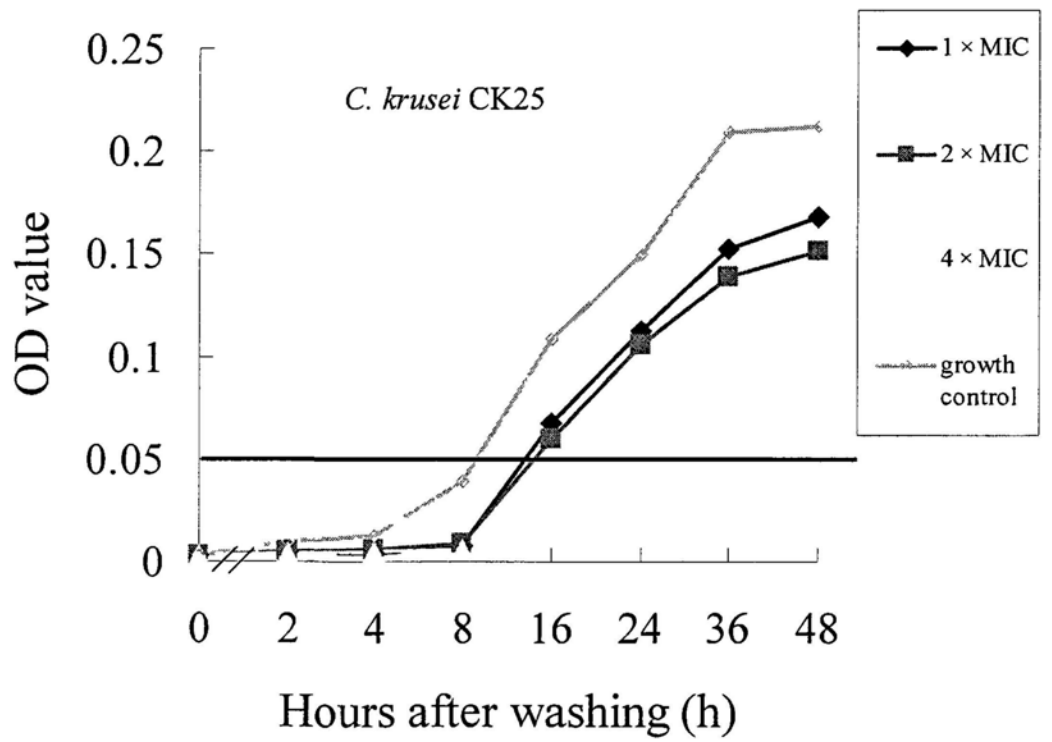


Fig. 4.4 The growth curves of *C. krusei* cells following 1-h exposure to metergoline. The PAFE was determined by the time of each group to reach the 0.05 absorbance level after washing.

4.2.5 Effect of metergoline on phospholipase production

Metergoline reduced the Pz values of the two *C. krusei* isolates after 2 h of incubation at 37 °C (Table 4.4). The Pz values were diminished by ~10% at 2 μg/ml of metergoline and by >20% at 8 μg/ml of metergoline.

Table 4.4 Pz values of *C. krusei* isolates after administration of metergoline at different concentrations for 2 h.

C. krusei isolates	Metergoline (μg/ml)			
	2	4	8	Control
ATCC 6258	0.620 ± 0.042 (10.71%)	0.630 ± 0.011* (12.50%)	0.690 ± 0.002** (23.21%)	0.560 ± 0.004
CK25	0.640 ± 0.036 (10.34%)	0.660 ± 0.011* (13.79%)	0.710 ± 0.025* (22.41%)	0.580 ± 0.012

* p <0.05, ** p <0.01. Numbers in parentheses represent percent reduction of extracellular phospholipase production with respect to the control (n=3).

4.2.6 Effect of metergoline on ROS production in *C. krusei*

In the present study, the fluorescent dye DCFDA was used to examine the changes in intracellular ROS level of *C. krusei* after metergoline treatment. The intracellular ROS level significantly increased with the concentration of metergoline. The increase was ~ 60% at 4 μg/ml and ~ 80% at 16 μg/ml. However, in the presence of ascorbic acid or mannitol, known ROS scavenger, such increase disappeared (Fig. 4.5). The MIC values of metergoline against *C. krusei* were also increased significantly by at least 4-fold in the presence of the scavenger (Table 4.5), showing that the antifungal activity of metergoline was ROS-dependent.

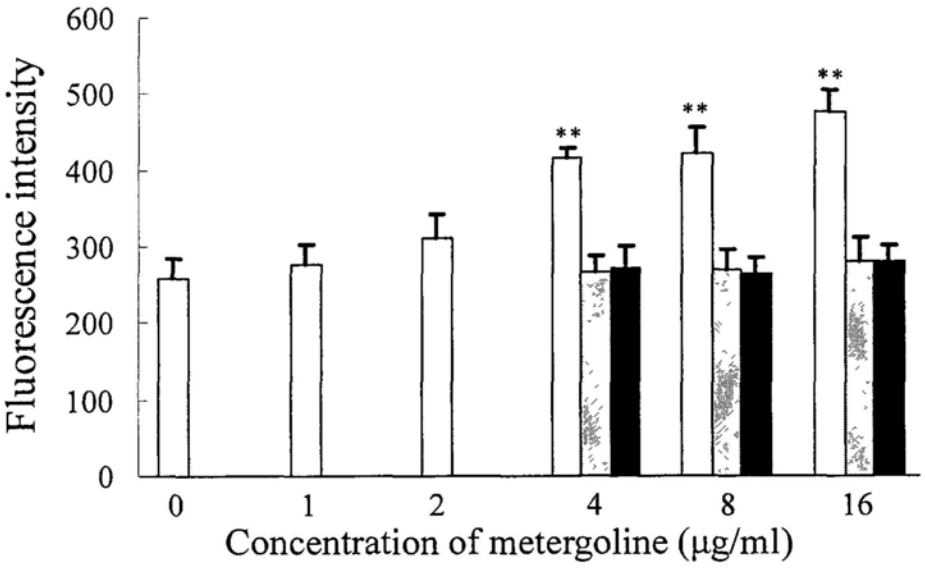


Fig. 4.5 Changes in intracellular ROS level in *C. krusei* ATCC 6258 after metergoline treatment (0 to 16 μg/ml), in the presence of 100 mM ascorbic acid (▨) or mannitol (■), as determined by DCFDA assay. The data were expressed as mean ± S.D. ** $p < 0.01$ when compared with the control.

Table 4.5 The antifungal activities of metergoline against *C. krusei* ATCC 6258 in the presence of ascorbic acid or mannitol.

C. krusei	MIC (μg/ml)		
	Metergoline	+ Ascorbic acid (100 mM)	+ Mannitol (100 mM)
ATCC 6258	4	>16	16

4.2.7 Effect of metergoline on MMP depolarization in *C. krusei*

The effect of metergoline on MMP in *C. krusei* was examined by the fluorescent dye JC-1. Exposure of *C. krusei* to metergoline caused a depolarization of MMP, as shown by a gradual reduction in the fluorescence intensity ratio (FL-2/FL-1) with increasing concentrations of metergoline (Fig. 4.6 & Fig. 4.7). The MMP was decreased by ~18% at 4 μg/ml metergoline and by ~ 52% at 16 μg/ml metergoline.

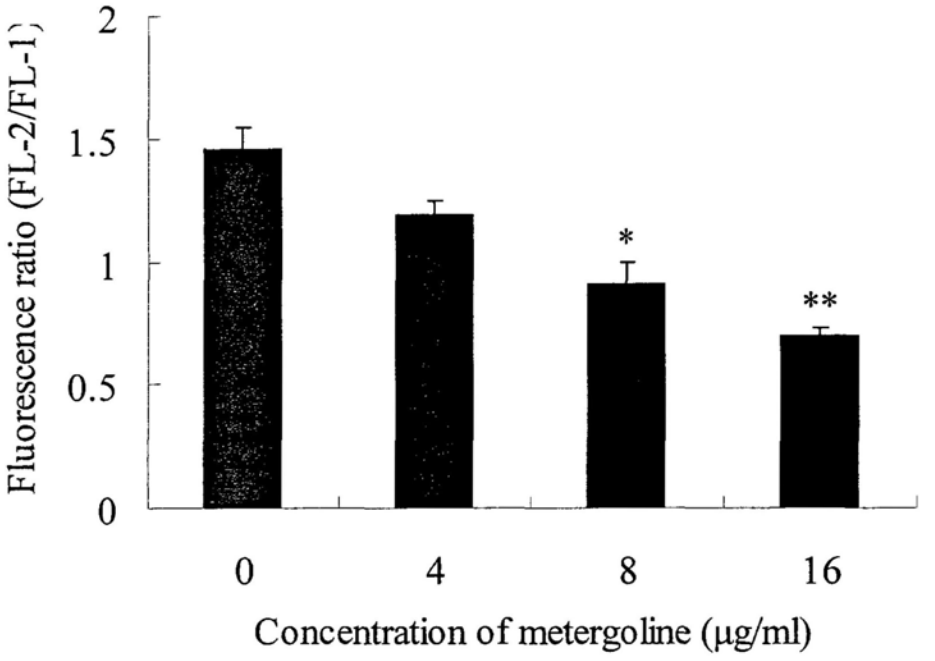


Fig. 4.6 Depolarization of MMP in *C. krusei* ATCC 6258 after metergoline treatment (0 to 16 μg/ml). The data were expressed as mean ± S.D. * $p < 0.05$, ** $p < 0.01$ when compared with the control.

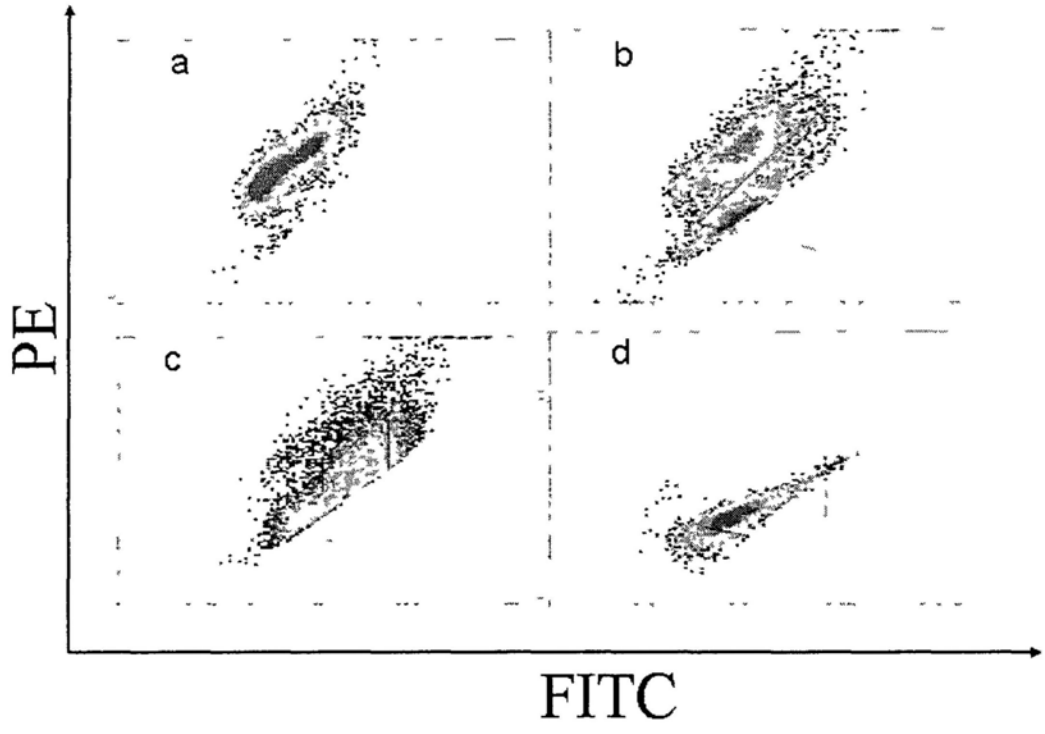


Fig. 4.7 A representative figure of the dot-plots, which shows a shift to the right and lower region, indicating a depolarization of mitochondrial membrane potential after treating with (a) 0, (b) 4, (c) 8 and (d) 16 μg/ml metergoline.

4.2.8 Metergoline induced apoptosis in *C. krusei*

The induction of apoptosis was monitored by flow cytometry using Annexin V/PI staining. After treatment with 4 μg/ml metergoline for 12 h, 5.6% of cells were apoptotic (Fig. 4.8). When the treatment dosage was increased to 8 μg/ml, nearly 29% of cells were apoptotic. Another familiar apoptotic feature is chromatin condensation and fragmentation. Metergoline elicited significant DNA fragmentation in *C. krusei* and ~90 % fungal cells were apoptotic at 16 μg/ml (Fig. 4.9a & Fig. 4.9b).

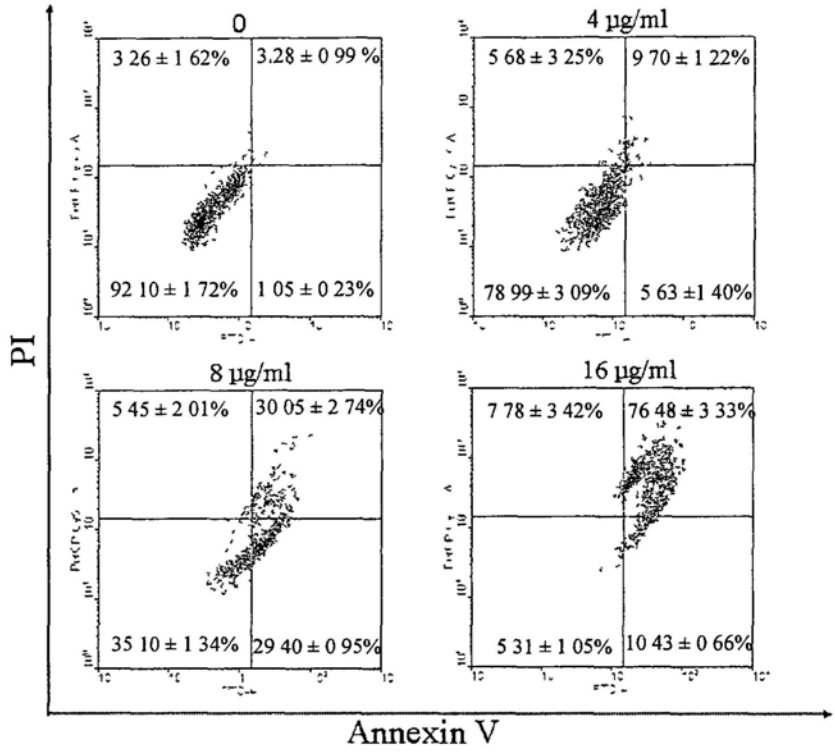
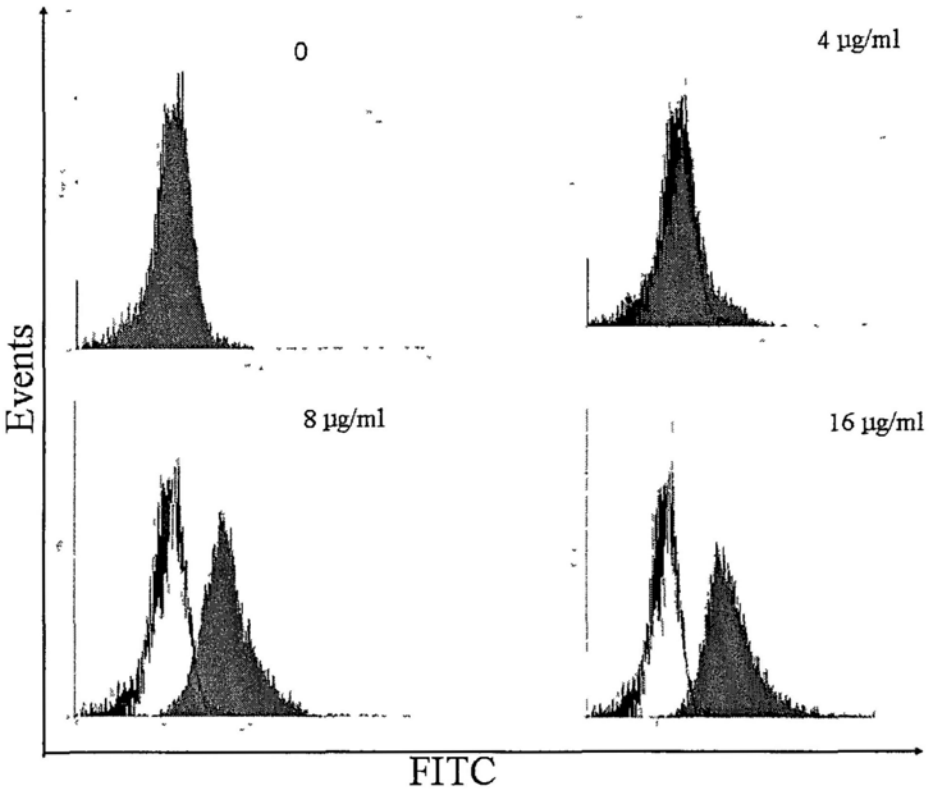


Fig. 4.8 Representative figures of the experiment on induction of apoptosis by metergoline (0 to 16 µg/ml) detected by flow cytometry using annexin V and PI. The data was expressed as mean ± SD (n=3).

A



B

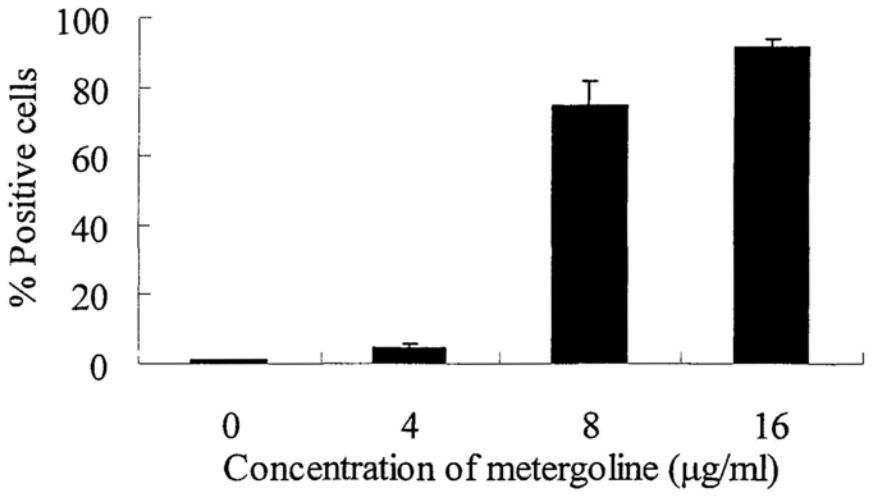


Fig. 4.9 Measurement of DNA fragmentation after metergoline treatment (0 to 16 µg/ml) using TUNEL assay. The data were analyzed with the WinMDI 2.9 software. A: Representative figures of the experiment. B: Mean value of the positive cell percentage of each group comparing to the blank control. Experiment was performed for three independent times. The data were expressed as mean ± SD (n=3).

4.3 Discussion

Emergence of non-*albicans Candida* infections is attributed to the growing body of compromised host groups such as AIDS patients and organ transplant patients (Pfaller *et al.*, 2008; Conen *et al.*, 2008; Muñoz *et al.*, 2005). Moreover, clinicians have encountered new challenges with these non-*albicans* species such as *C. krusei* owing to their inherent antifungal resistance to commonly used antifungal agents such as polyenes and azoles (Prasad and Kapoor, 2005; Pfaller *et al.*, 2008). Therefore, there is an urgent need to develop novel antifungal agents. Recent studies have focused on developing novel antifungal agents from various sources (Cederlund and Mardh, 1993). In these studies, several non-antibiotic substances have been shown to possess good antifungal activity *in vitro* (Cederlund *et al.*, 1993; Qiao *et al.*, 2007). Through screening a compound library, we found that metergoline, a serotonin receptor antagonist, possessed antifungal activity, especially against *C. krusei* isolates.

Metergoline is a serotonin-selective antagonist which can effectively inhibit several 5-HT receptor subtypes at low dose, especially for 5-HT_{2A} and 5-HT_{2C} receptor. At low doses, it has no effects on the GABA or dopamine neurotransmitter systems and has been shown to be relatively well tolerated (Fuxe *et al.*, 1975; Greenberg *et al.*, 1998).

Several articles reported that serotonin and its reuptake inhibitors have been suggested as potential novel antifungal candidates due to their efficacy against *Candida* and *Aspergillus* species (Lass-Flörl *et al.*, 2001; Mayr *et al.*, 2005;

Lass-Flörl *et al.*, 2003b). At present, no fungal orthologue of serotonin receptor is known to exist in *Candida* species and therefore metergoline may exert its antifungal effects via interaction with some other cellular targets or mechanisms, which have also been confirmed by the results of the weak antifungal activities of the six serotonin receptor antagonists studied.

Metergoline exhibited a considerable antifungal activity against *C. krusei* isolates. In addition to the ATCC strain, we also tested the susceptibility of another 13 wild-type isolates of *C. krusei*, and the same values of MIC and MFC were obtained. Interestingly, combination study demonstrated that metergoline exerted potent synergism in combination with AMB at subinhibitory concentrations against the two *C. krusei* isolates. Although synergistic activity of metergoline with FLC was not consistent, the MIC values of FLC were lower when combining them together.

Combination of antifungal agents is widely used to provide broad-spectrum coverage in the treatment of complex fungal infections. It reduces the likelihood of developing drug resistance and decreases dose-related toxicity (Prasad and Kapoor, 2005). *In vitro* synergisms of antifungal agents have been observed in treating *C. albicans* (Marchetti *et al.*, 2000; Guo *et al.*, 2008; Huang *et al.*, 2008). In a case report, combination therapy with AMB and caspofungin successfully treated a leukemic patient with *C. krusei* fungemia who failed to respond to liposomal AMB therapy alone, which provided a good example of combination therapy be a useful treatment option for invasive candidiasis, particularly when caused by more resistant species (Olver *et al.*, 2006). Polyenes and azoles are the most widely applied classes of antifungal agents, and FLC has been shown to be as effective as A MB in the

treatment of candidemia. So the combination study is of high relevance in clinical practice. Our results showed that metergoline could be successfully combined with either AMB or FLC to provide synergistic antifungal activity against *C. krusei in vitro*. FLC inhibits lanosterol 14α-demethylase, a crucial enzyme in ergosterol biosynthesis and AMB decreases membrane ergosterol content, causing disruption of fungal membrane (Prasad and Kapoor, 2005). Although the mechanism of this synergistic activity is not totally clear at present, it could be surmised that weakening of fungal membrane structures by metergoline to increase cell permeability may facilitate the easier penetration of either FLC or AMB to *C. krusei*, thus augmenting antifungal activity.

Metergoline has shown potent anti-fungal effect on *C. krusei* isolates. However, a good antifungal agent should have selectivity and not harmful to mammalian cells. To examine the cytotoxicity of metergoline, MTT assay was used. Colorimetric assays of cell viability are important tools to study the eukaryotic cell activity. A mainstay of such techniques is involving the use of tetrazolium salts (such as MTT) (Altman, 1976). The results showed that although the selectivity was not very high, the IC_{50} value against the mammalian cell lines was higher than the antifungal MIC value. Having the synergistic activities with AMB and FLC, when using as a supplement to treat candidiasis, metergoline may have lower cytotoxicity.

The reagents used in the colorimetric assays have evolved from MTT to XTT. The later has proved its special advantage over MTT as XTT is reduced more efficiently than MTT and the formazan product is water soluble, enabling the simplification of assay performance (Kuhn *et al.*, 2003). Due to the ease of use, XTT has been used

for fungal susceptibility testing (Hawser *et al.*, 1998). It is anticipated that XTT method could be used to study fungal growth and drug susceptibility. XTT is converted to a colored formazan in the presence of metabolic activity, mainly by the mitochondrial succinoxidase, cytochrome P450 systems and flavoprotein oxidases (Altman, 1976). Using XTT to determine the fungal activity showed a direct relationship between colormetric signal and cell number (Hawser, 1996). Metergoline could inhibit the fungal growth and the cell viability. The XTT results were consistent to the previous MIC value which was determined as 80% inhibition by OD value.

PAFE denotes the suppression of fungal growth after limited exposure to antifungal agents. In practice, the goal of treatment against infection is to maintain the concentration of antifungal agents above the MIC value for the entire dosing period; however, it is questionable whether it can be achieved intra-orally due to many factors. Therefore, in treating candidal infections, it would be desirable if the clinicians could rely on *in vitro* susceptibility tests to obtain critical information. The PAFE assay may provide clinical relevance in the design of dosing regimens, such as the frequency of administration of the antifungal agents (Kustimur *et al.*, 2003; Uchida *et al.*, 2006; Scalarone *et al.*, 1991; Rex *et al.*, 1993). Metergoline was defined as a fungicidal antifungal agent, whose MFC value was two times of the MIC value. This was also confirmed by the characteristic of the PAFE curve.

Production of extracellular phospholipase is a well-known virulence factor in *Candida* which degrades cell membrane of host tissue (Kantarcioğlu and Yücel, 2002; Anil and Samaranayake, 2003). Thus, it has been shown that adherence and

penetration of *Candida* to the host tissues are correlated with phospholipase activity (Ghannoum 2000). *C. krusei* isolates used in the present study showed high extracellular phospholipase production (Pz value <0.63). Intriguingly, metergoline reduced the phospholipase production after 2 h of incubation at 37 °C. More interestingly, a recent study on the antileishmanial activity of sertraline suggested a linkage between drug-induced apoptosis and inhibition of phospholipase enzymes (Palit and Ali, 2008). Therefore, it could be hypothesized that metergoline may initiate apoptosis in *C. krusei*.

Apoptosis, or programmed cell death, is a well-established phenomenon that has been observed as a highly organized cellular process leading to cell death in multicellular organisms and is indeed required for normal growth, development and cell maintenance. Recent studies also confirmed the existence of apoptosis in baker's yeast and *C. albicans* (Phillips *et al.*, 2003; Phillips *et al.*, 2006; Wissing *et al.*, 2004; Madeo *et al.*, 1997; Madeo *et al.*, 2004; Ramsdale, 2008). Apoptosis or signs of apoptosis in *Saccharomyces cerevisiae* can be induced by mutation in *CDC48* and by external stimuli such as salt stress and ultraviolet radiation (Wissing *et al.*, 2004). Yeast orthologues of mammalian counterparts that regulate apoptosis have been identified, implying phylogenetic conservation of cell death pathways (Wissing *et al.*, 2004; Madeo *et al.*, 2002). Recent evidence has demonstrated the existence of apoptosis in *C. albicans* after exposure to quorum-sensing molecule farnesol, acetic acid, hydrogen peroxide and low doses of AMB (Phillips *et al.*, 2003; Phillips *et al.*, 2006). Activation of the Ras-cAMP-PKA pathway can also speed up apoptosis in *C. albicans* (Phillips *et al.*, 2006).

Observed features of apoptosis include decreased cell size, chromatin condensation and nuclear fragmentation due to endonuclease cleavage of DNA. Apoptosis involves many factors such as apoptosis-inducing factor, yeast metacaspase, messenger RNA decapping associated gene, inositolphosphosphingolipid phospholipase, and Ras pathway signaling (Almeida *et al.*, 2008; Guaragnella *et al.*, 2006; Mazzoni *et al.*, 2005; Phillips *et al.*, 2006; Wissing *et al.*, 2004). Mitochondria not only function as a key determinant of cell life, but also participate in yeast programmed cell death (Ludovico *et al.*, 2002). As mitochondria are essential organelles for cellular physiology and integrity, critical metabolic processes might be affected and led to growth inhibition and/or cell death in *Candida* isolates. During the apoptotic process, cytochrome c is released from mitochondria into the cytoplasm as a key event, through binding of the released cytochrome c to apoptotic protease activating factor-1 (Apaf-1)-procaspase-9 complex, following effector caspase-3 activation and DNA fragmentation (Srinivasula *et al.*, 1998; Zou *et al.*, 1997). Mitochondria play a pivotal role in *Candida* apoptosis as depolarization of MMP resulted in the release of mitochondrial proteins and activation of caspases, initiating the cell death scenario. The MMP is a direct indicator of mitochondrial function, whose breakdown is associated with apoptosis in *Candida* species and disturbance in the MMP has been considered as a signal for the onset of apoptosis (Wang and Youle, 2009). Metergoline depolarized MMP, which indicated that it may induce apoptosis in *C. krusei*.

Intracellular oxygen radicals are mainly generated in mitochondria. ROS can damage almost every essential cellular component, resulting in enzyme inactivation, membrane disruption and ultimately cell death (Shirtliff *et al.*, 2009). ROS play a

central role in promoting oxidative stress and may trigger apoptotic or programmed cell death processes (Wunder *et al.*, 2004). The importance of ROS as apoptosis inducer has been demonstrated in cells. High ROS levels might be associated with the presence of apoptotic markers, although a causative effect has yet to be demonstrated. In somatic cells, targeting antioxidants to the mitochondria can prevent the loss of MMP and apoptosis, suggesting that ROS is a good indicator to show the occurrence of apoptosis (Simon *et al.*, 2010; Martinez-Pastor *et al.*, 2009; Pozniakovsky *et al.*, 2005). Depolarization of the MMP is followed by an elevation of the intracellular ROS level. ROS are detrimental to cell viability, resulting in enzyme inactivation, membrane disruption and cell death (Kobayashi *et al.*, 2002). ROS production in *C. krusei* was significantly increased after being treated with metergoline, which was confirmed by the facts that adding ascorbic acid and mannitol could scavenge the ROS. The MIC values of metergoline against *C. krusei* were increased significantly by at least 4-fold in the presence of the scavenger, showing that the antifungal activity of metergoline was ROS-dependent.

Changes in the phospholipid bilayers of cell membranes are observed early in apoptosis. The phosphatidylserine (PS) component of the phospholipid bilayers are translocated from the inner (cytoplasmic) leaflet of the plasma membrane to the outer (cell surface) leaflet after the induction of apoptosis, and can be detected by fluorescence labeling. Annexin V is a member of the annexin family of calcium-dependent phospholipid-binding proteins and has a strong, specific affinity for PS-containing phospholipid bilayers. Staining with FITC-conjugated annexin V and PI can identify subpopulations of cells with membrane changes and the associated loss of membrane integrity (Dachary-Prigent *et al.*, 1993; Shinbrot *et al.*,

2000; Yang *et al.*, 1996; Zhang *et al.*, 1997). After being treated with metergoline, *C. krusei* protoplasts were detected by annexin V/PI using flow cytometry and the results showed that the proportion of annexin (+) PI(–) was increasing with metergoline concentration. However, due to the existence of cell wall in *Candida* species, there is a need of enzyme to digest it. This may result in some damage to cell viability and increase cell death. So there may be some errors in the results. Anyway, our results showed that metergoline induced apoptosis in *C. krusei*.

Later steps of the mitochondrial-dependent cell death pathway involve the translocation of apoptosis-inducing factor from the mitochondria to nucleus, leading to chromatin condensation and DNA cleavage (Madeo *et al.*, 2004; Ramsdale, 2008). During apoptosis, chromatin DNA is cleaved into inter-nucleosomal fragments and one of the characteristics of apoptosis is the fragmentation of DNA. There are many ways to detect apoptosis at different stages. The most commonly used method is TUNEL assay. The TUNEL assay can identify apoptotic cells *in situ* using terminal deoxynucleotidyl transferase to transfer biotin-dUTP to these strand breaks of cleaved DNA. The biotin-labeled cleavage sites are then detected by reaction with horseradish peroxidase. We assessed the effect of metergoline on DNA integrity by the TUNEL assay to measure the free 3'-OH termini in apoptotic DNA ends using fluorescently tagged nucleotide (FITC-labeled deoxyuridine triphosphate). The results showed that metergoline could increase DNA fragmentaion in *C. krusei*. However, false positive staining in the TUNEL assay to detect apoptosis may occur. For example, when applied to liver tissue, the false signal is caused by the release of endogenous endonucleases as a result of proteinase treatment (Stähelin *et al.*, 1998). This can be abolished by pretreatment of tissue with diethyl pyrocarbonate. Recently,

more methods for the detection of apoptotic cells are available such as the detection of caspase activity, fas-ligand and annexin V, etc. Thus, it is very important to combine TUNEL with these assays to confirm the process of apoptosis (Stähelin *et al.*, 1998).

Taken together, the present study for the first time demonstrated the potent *in vitro* antifungal activity of metergoline against FLC-resistant *C. krusei* isolates. We have found that dying *C. krusei* cells exhibited many key apoptotic markers. After exposure to metergoline, *C. krusei* displayed viability decrease, MMP depolarization. Furthermore, DNA damage exposing free 3'-OH groups was detected by the TUNEL assay. Intracellular ROS levels were increased significantly after metergoline treatment. Finally, plasma membrane translocation of PS was detected on the outer surface. All these evidence confirmed the apoptosis pathway of the antifungal activity of metergoline. Chemical-induced cell death in fungi is intricate and further genome-wide experiments are needed to investigate the molecular mechanism of metergoline and to harness its antifungal potential, particularly targeting *C. krusei* - a notorious yeast species resistant to conventional drugs in clinical settings.

Chapter 5

Antifungal Activity of Purpurin against *Candida* Species

5.1 Introduction

Extracts and constituents from plant materials are considered as unlimited sources of novel biomolecules with diverse functions. Bioactive compounds have been identified from plant materials with antiviral, antibacterial, antifungal and anticancer activities (Ríos and Recio, 2005). Anthraquinones are commonly found in higher plants. A number of reports have shown the antimalarial, antibacterial and antifungal activities of anthraquinones. In particular, emodin, alizarin, chrysophanol and rhein exhibit antifungal activity against *C. albicans* (Agarwal *et al.*, 2000; Kong *et al.*, 2009; Xiang *et al.*, 2008). In the present study, we reported the novel antifungal activity of purpurin, a natural anthraquinone pigment currently used as food colouring agent and for cotton printing, against *Candida* species. To gain insight into the underlying antifungal mechanisms of purpurin, we examined the effect of purpurin on efflux pumps and mitochondria. Our results indicated that purpurin inhibited energy-dependent membrane efflux pumps and depolarized MMP in *Candida* species. Dissipation of the MMP has been attributed to initiation of cell death pathway, implying the antifungal activity of purpurin may be related to fungal apoptosis.

5.2 Results:

5.2.1 *In vitro* susceptibility to purpurin

The antifungal activity of purpurin on *Candida* isolates was evaluated by a broth microdilution assay and a summary of the MIC values is shown in Table 5.1. Purpurin exhibited potent *in vitro* antifungal activity against all *Candida* species (MIC range = 1.28-5.12 μg/ml), especially on *C. krusei* and *C. kefyr* isolates (MIC = 1.28 μg/ml).

Table 5.1 The MIC values of purpurin and FLC against *Candida* species after 24 h of incubation at 35 °C

Candida isolates	MIC (μg/m l)		*Candida* isolates	MIC (μg/m l)	
	Purpurin	FLC		Purpurin	FLC
C. krusei ATCC 6258	1.28	32	*C. albicans* CA12	5.12	1.0
C. krusei CK1	1.28	32	*C. albicans* CA71929	5.12	0.5
C. krusei CK3	1.28	32	*C. albicans* CA194	2.56	1.0
C. krusei CK5	1.28	64	*C. albicans* SC5314	5.12	1.0
C. krusei CK9	1.28	64	*C. tropicalis* ATCC 13803	5.12	0.5
C. krusei CK13	1.28	64	*C. tropicalis* 90/1362	5.12	2.0
C. krusei CK15	1.28	32	*C. parapsilosis* ATCC 22014	5.12	2.0
C. krusei CK17	1.28	64	*C. parapsilosis* 90/2224	2.56	2.0
C. krusei CK18	1.28	64	*C. glabrata* ATCC 90030	2.56	8.0
C. krusei CK25	1.28	64	*C. glabrata* WT-5	2.56	8.0
C. albicans ATCC 18804	5.12	0.5	*C. kefyr* 199	1.28	0.5
C. albicans ATCC 18804	2.56	0.5	*C. kefyr* 219	1.28	0.5

5.2.2 Purpurin enhanced the potency of FLC against *C. krusei*

The interaction study showed that there was no synergistic effect between purpurin and FLC (FICI: 0.625–1.125) against 10 *C. krusei* isolates (Table 5.2). However, combinational treatment against the *C. krusei* isolates together with purpurin could decrease the MIC values of FLC. For example, when *C. krusei* ATCC 6258 was treated by FLC and 0.64 μg/ml purpurin, the MIC value of FLC decreased to 4 μg/ml (1/8 of the MIC alone). The combination data suggested that purpurin could intensify the potency of FLC.

Table 5.2 The *in vitro* interaction between purpurin and FLC against *C. krusei* isolates after 24 h incubation at 35 °C

C. krusei isolate	Purpurin (μg/ml)	FLC (μg/ml)	FICI*
	MIC alone	MIC alone	Purpurin + FLC
ATCC 6258	1.28	32	0.625[a]; (0.64/4)[b]
CK1	1.28	32	0.625[a]; (0.64/4)[b]
CK3	1.28	32	0.750[a]; (0.64/8)[b]
CK5	1.28	64	0.625[a]; (0.64/8)[b]
CK9	1.28	64	1.125[a]; (1.28/8)[b]
CK13	1.28	64	0.625[a];(0.64/8)[b]
CK15	1.28	32	0.750[a]; (0.64/8)[b]
CK17	1.28	64	1.125[a]; (1.28/8)[b]
CK18	1.28	64	0.625[a]; (0.64/8)[b]
CK25	1.28	64	0.750[a]; (0.64/16)[b]

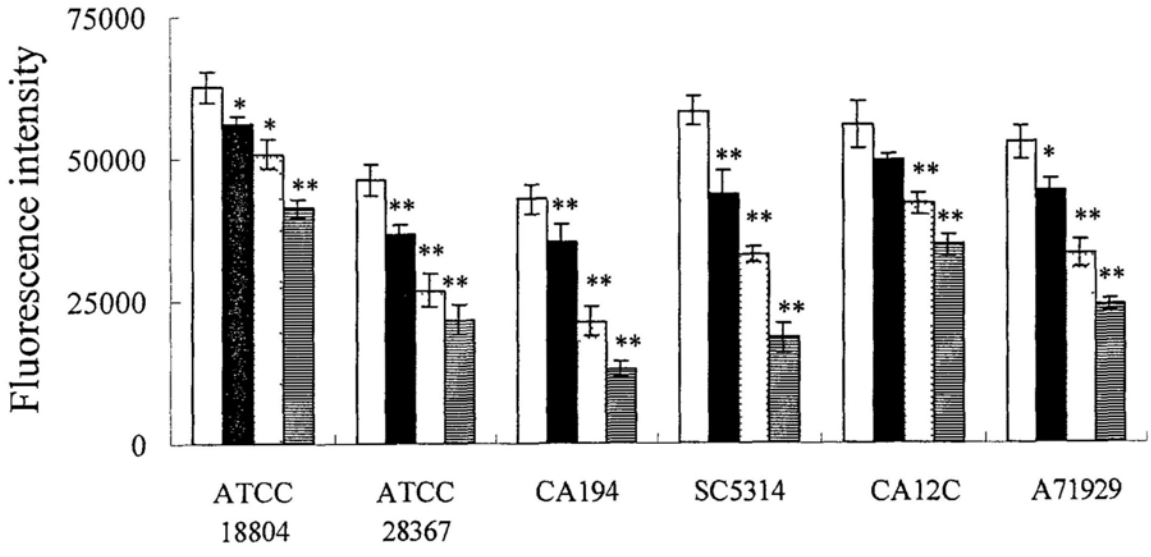
* FICI= the sum of the MIC of each compound when used in combination divided by the MIC of the compound alone.

[a] Indifferent, [b]MIC values (μg/ml) of each compound in combination

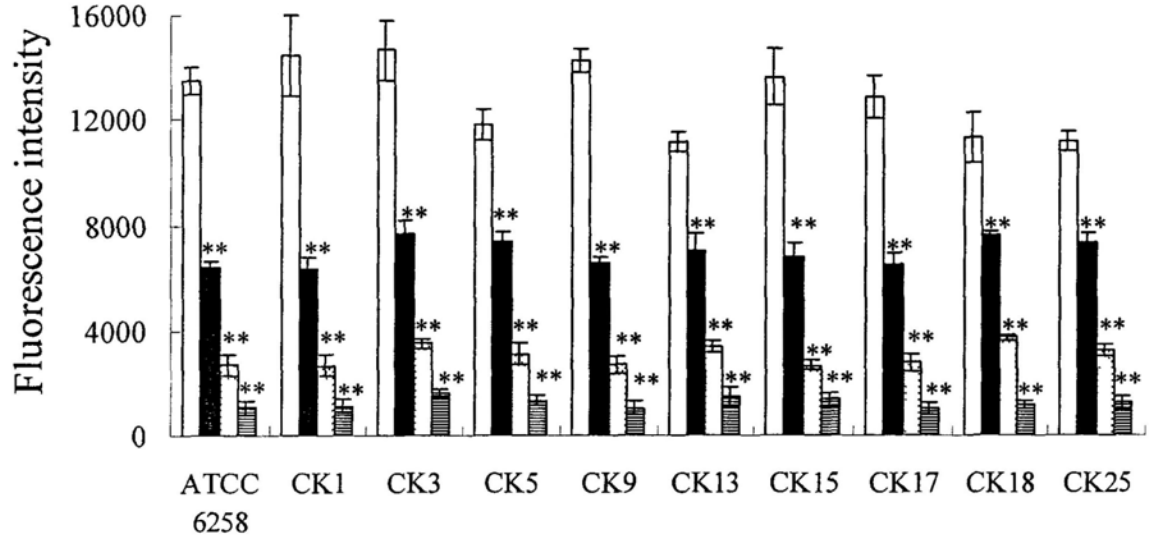
5.2.3 Purpurin inhibited the efflux of R6G

Purpurin strongly inhibited the energy-dependent efflux pumps of *Candida* isolates (Fig. 5.1). The inhibition was reflected by a decreasing level of R6G in the supernatant of fungal cells after exposure to increasing concentrations of purpurin.

(A)


Fluorescence intensity
75000
50000
25000
0
ATCC 18804
ATCC 28367
CA194
SC5314
CA12C
A71929

(B)


Fluorescence intensity
16000
12000
8000
4000
0
ATCC 6258
CK1
CK3
CK5
CK9
CK13
CK15
CK17
CK18
CK25

(C)

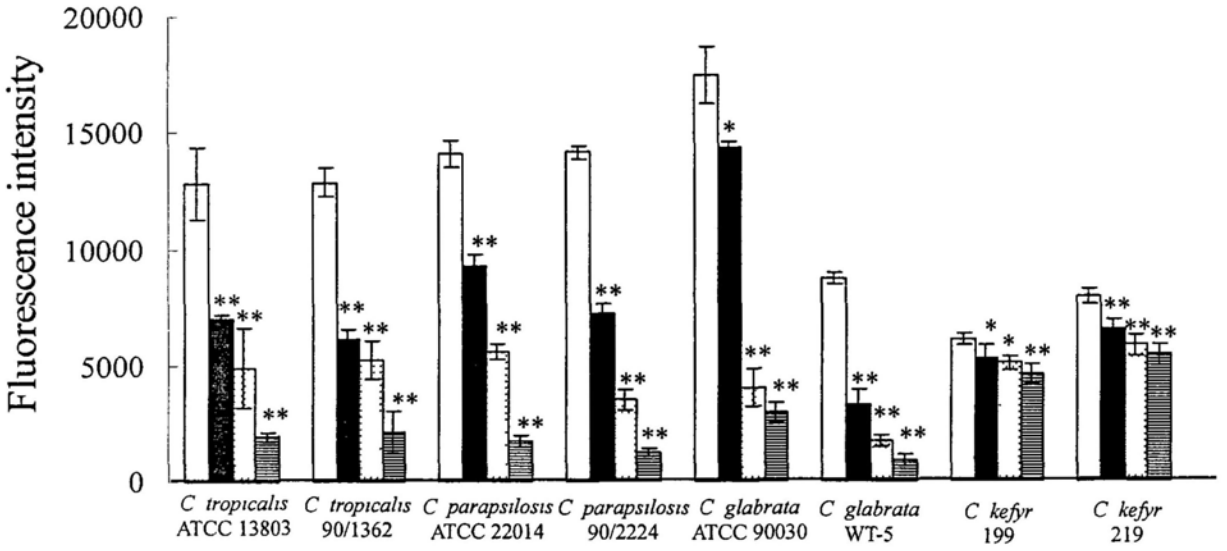
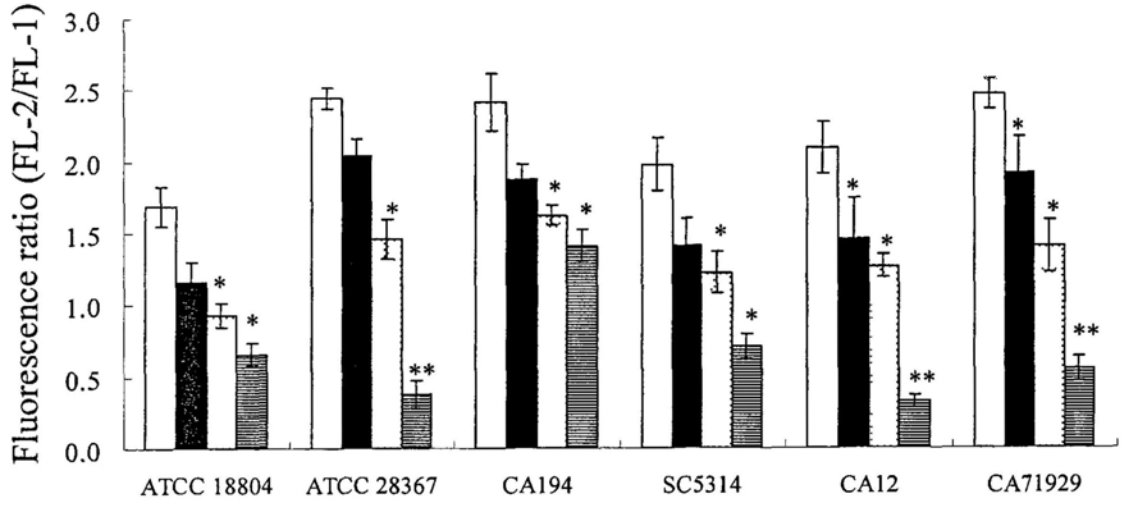


Fig. 5.1 Determination of the effect of purpurin on the activity of energy-dependent efflux pumps of (A) *Candida albicans*, (B) *Candida krusei* and (C) other species isolates by using R6G extrusion assay. R6G efflux from the fungal cells was determined by measuring the fluorescence intensity of the supernatant (excitation wavelength: 485 nm; emission wavelength: 535 nm). Purpurin concentrations: 0 (□), 1 × MIC (■), 2× MIC (□) or 4× MIC (▤). The data were expressed as mean ± SD. * $p < 0.05$, ** $p < 0.01$ when compared with the respective controls (n = 3).

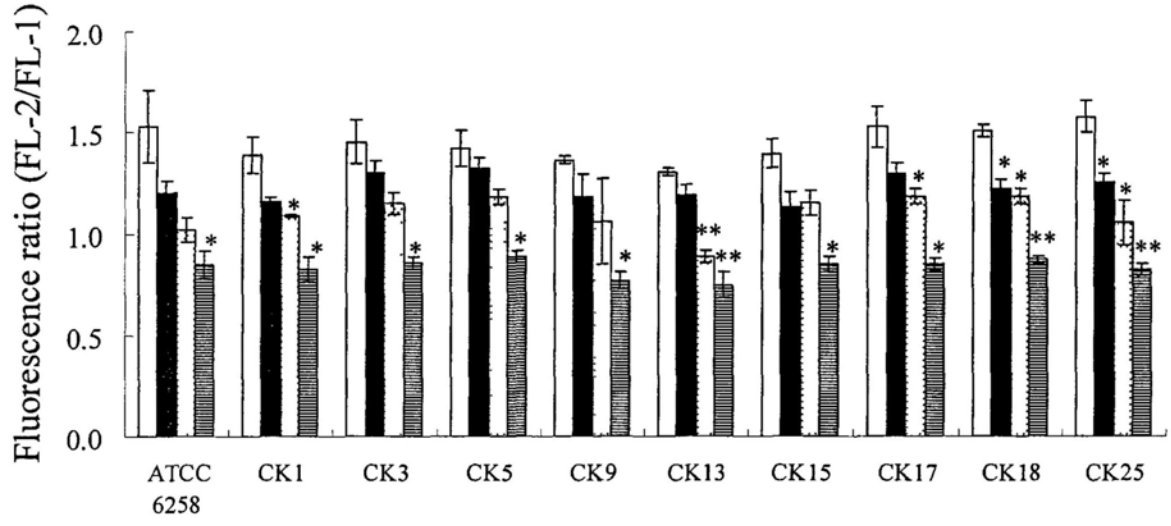
5.2.4 Purpurin depolarized mitochondria in *Candida* species

To examine the effect of purpurin on mitochondria, the MMP of purpurin-treated fungal cells was determined by the fluorescent dye JC-1. Depolarization of MMP was revealed by a progressive reduction in the fluorescence intensity ratio (FL-2/FL-1) with increasing concentrations of purpurin (Fig. 5.2).

(A)


Fluorescence ratio (FL-2/FL-1)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ATCC 18804
ATCC 28367
CA194
SC5314
CA12
CA71929

(B)


Fluorescence ratio (FL-2/FL-1)
2.0
1.5
1.0
0.5
0.0
ATCC 6258
CK1
CK3
CK5
CK9
CK13
CK15
CK17
CK18
CK25

(C)

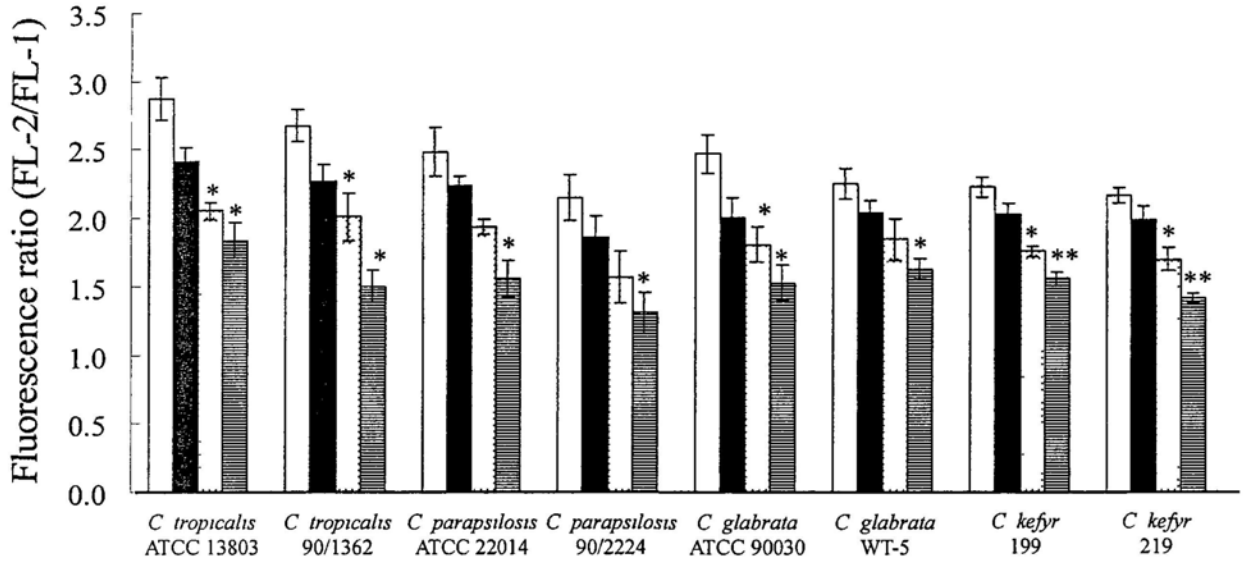


Fig. 5.2 Depolarization of MMP by purpurin in (A) *Candida albicans*, (B) *Candida krusei* and (C) other species isolates. The fluorescence of JC-1 was measured by Beckman Coulter flow cytometer at FL-1 (525 nm) and FL-2 (595 nm) and the MMP was expressed by the ratio of FL-2/FL-1. Purpurin concentrations: 0 (□), 1 × MIC (■), 2 × MIC (⬚) or 4 × MIC (▒). The data were expressed as mean ± SD.

* $p < 0.05$, ** $p < 0.01$ when compared with the respective controls (n = 3).

5.3 Discussion

A key issue of rapidly growing global concern was drug resistance and epidemiological studies indicated the emergence of resistant *Candida* strains in clinical settings. Pathogenic fungi have several defence mechanisms. Amino acid substitutions (e.g. Y132H, G464S, and R467K) in cytochrome P450 lanosterol 14α-demethylase have been identified to confer resistance to azoles and mutations at S645 of β-1,3-glucan synthase have been correlated with resistance to echinocandins (Carrillo-Muñoz *et al.*, 2006). The simplest mechanism of resistance is reducing intracellular drug accumulation which will lead to a reduction in the amount of drug that can reach a target. Multidrug resistance in fungi can be mediated by overexpression of efflux pumps, the energy-dependent ATP-binding cassette (ABC) transporters and the proton gradient-driven major facilitators, in several *Candida* species and other pathogenic fungi (Del Sorbo *et al.*, 2000). For example, effective inhibition of P-glycoprotein-mediated efflux will increase intracellular drug concentration in P-glycoprotein-expressing cells to levels comparable to those found in cells that do not express P-glycoprotein (Fojo and Bates, 2003). Recently, some transcription factors, such as MRR1 (multidrug resistance regulator) and TAC1 (transcriptional activator of CDR genes), that were coordinately upregulated with MDR1 (multidrug resistance), were identified as the central regulators of the MDR1 efflux pump (Morschhäuser *et al.*, 2007; Coste *et al.*, 2004). Furthermore, evidence shows an increasing number of non-*albicans Candida* infections, among which *C. krusei* infection is of paramount importance owing to its innate resistance to FLC and reduced susceptibility to AMB and flucytosine (Samaranayake, 1997; Krcmery and Barnes, 2002), which has been proposed to be due to the low affinity of cytochrome

P450 lanosterol 14α-demethylase for FLC and the constitutive expression of the efflux pump (Lamping *et al.*, 2009). The innate FLC resistance of *C. krusei* may account for the dramatic increase in the number of *C. krusei* infections (Pfaller *et al.*, 2008). The mortality rate of *C. krusei* infections in patients with hematologic malignancies and transplantations can reach 60% (Muñoz *et al.*, 2005)! Although *C. krusei* is susceptible to voriconazole and caspofungin, failure in treatment has been reported in clinical settings (Hakki *et al.*, 2006; Pelletier *et al.*, 2005). In light of these considerations, discovery and characterization of novel antifungal agents and targets are top priorities in *Candida* research and extensive efforts are focusing on developing antifungal agents with characteristic of reversing multidrug resistance.

Plant materials are believed to be an unlimited source of novel biomolecules. A number of potent bioactive compounds have been isolated from the screening of natural products (Hakki *et al.*, 2006; Pelletier *et al.*, 2005; Ríos and Recio, 2005; Agarwal *et al.*, 2000). Anthraquinones, commonly found in higher plants, have been shown to exhibit diverse biological functions. Besides anticancer and antimalarial activities, some anthraquinones have been documented to possess antibacterial and antifungal activities. In the present study, we demonstrated for the first time that purpurin (1,2,4-trihydroxy-9,10-anthraquinone), a natural anthraquinone red pigment from madder root (*Rubia tinctorum* L.), possesses potent antifungal activity against *C. albicans* and *C. krusei* isolates. Prior biochemical studies of purpurin have focused on its antimutagenic effect on heterocyclic amines (Marczylo *et al.*, 1999) and carcinogens (Takahashi *et al.*, 2002). The beneficial chemopreventive potential of purpurin has been attributed to inhibition of cytochrome P450-dependent metabolism of harmful substances.

Drug efflux pumps are ubiquitously present in all living cells, including bacteria, fungi and cancer cells. A major drug resistance mechanism in *Candida* species is mediated by the overexpression of membrane-bound efflux pumps. Efflux pumps reduce drug accumulation by transporting drugs out of the cells and their overexpression is linked up with the multidrug-resistant phenotype in several *Candida* species and pathogenic fungi (Piddock, 2006; Gatti *et al.*, 2009). Efflux pump may be specific for one substrate or may transport a range of structurally dissimilar compounds. There are several major families of efflux transporter, for example, major facilitator, multidrug and toxic efflux pump and ATP binding cassette. All bacterial and fungus genomes studied contain several different efflux pumps (Webber and Piddock, 2003). The efflux pumps of *C. albicans* (Cdr1p and Cdr2p) and *C. krusei* (Abc1p) are energy-dependent where the constitutive expression of the latter has been attributed to the innate FLC resistance of *C. krusei* (Lamping *et al.*, 2009). Compounds that inhibit efflux pump activity may have potential therapeutic value. Here, we demonstrated that purpurin strongly inhibited the activity of these efflux pumps. The exact type of efflux pump inhibited by purpurin is not clear. The further studies are needed to explore the inhibiting effect on efflux pump so as to understand the antifungal mechanism of purpurin. Although purpurin and FLC did not have synergistic effect, purpurin could reverse the FLC resistance to lower down the MIC values.

In one aspect of apoptosis, the onset of mitochondrial-dependent cell death pathway is manifested by depolarization of MMP. Subsequent release of cytochrome *c* and apoptosis-inducing factor from mitochondria to cytosol and nucleus causes protein aggregation, chromatin condensation and eventually cell death (Madeo *et al.*, 2004;

Ramsdale, 2008). We investigated the effect of purpurin on the MMP by using the cationic fluorescent probe JC-1 which exhibits a potential-dependent accumulation in mitochondria. Purpurin treatment depolarized the MMP was reflected by the reduction in the red/green fluorescence intensity ratio (FL-2/FL-1), implying a perturbation of mitochondrial homeostasis.

As a major clinical problem of drug resistance, conventional chemotherapeutic drugs encountered difficulty in treating infections. Considerable efforts have been made to circumvent this. One possible approach is the use of resistance modulators to reverse multidrug resistance and then sensitize resistant cells to antifungal agents. Agents evaluated since the early 1980s include: verapamil, quinidine, tamoxifen, cyclosporine A, dexverapamil, valspodar, tariquidar and others (Ford and Hait, 1990; Fisher *et al.*, 1996). Trials attempting to modulate drug resistance have undergone a gradual evolution from early trials using agents already approved by the FDA for other indications, to subsequent studies with more poten agents developed as inhibitors (Fojo and Bates, 2003). However, the drug-drug interaction resulted in disappointing and unpredictive results, such as the dose-limiting toxicity and pharmacokinetic disorder. The other approach is to develop novel antifungal agents bypassing multidrug resistance (Robert, 2001; Gottesman *et al.*, 2002). In our study, we demonstrated purpurin possessed potent antifungal activities against FLC-resistant strains with the ability of inhibiting the efflux pump, showing its promising use in treating clinical infections.

To the best of our knowledge, we reported here for the first time the novel antifungal activity of purpurin against *Candida* species. The antifungal activity of purpurin was

correlated to its inhibitory effect on the efflux pumps and the MMP. In the context of phylogenetic conservation of the core features of the cell death responses in different cell types (Wang and Youle, 2009; Ramsdale, 2008; Cao *et al.*, 2009), it can be postulated that similar apoptotic events can be induced in *C. krusei*. The reversal drug resistance mechanism of purpurin and its mode of action need to be further studied.

Chapter 6

Antifungal Activity of Baicalein against *Candida krusei*

6.1 Introduction

Traditional Chinese medicine (TCM) is believed to be an important source of novel bioactive compounds. Biomolecules have been identified from TCM with antiviral, antibacterial, antifungal and anticancer activities (Ríos and Recio, 2005). The dry root of *Scutellaria* is one of the most multi-purpose herbs used in China and the main bioactive component is flavone. The flavones have been confirmed to exert antiviral, antioxidant, anti-inflammatory and anti-cardiovascular illness (Gao *et al.*, 1999; Wu *et al.*, 2001; Guo *et al.*, 2007; Huang *et al.*, 2005; Chi *et al.*, 2003; Wang *et al.*, 2007). The anti-inflammatory activity of the flavones is partly due to their ability to suppress expression of monocyte chemotactic protein-1 (You *et al.*, 1999; Wakabayashi *et al.*, 2000). In addition to the anti-inflammatory activities, flavones have possessed cytotoxic activities against human cancer cell lines through scavenging oxidative radicals, attenuating NF-κB activity, inhibiting several genes important for regulation of the cell cycle, suppressing COX-2 gene expression and preventing viral infections (Min, 2009).

Baicalein, a water-insoluble flavone, has been reported to have antimicrobial activity and synergistic effect with other antibiotics (Huang *et al.*, 2008). It also possessed antifungal activity against *C. albicans* biofilms and induced programmed cell death in *C. albicans* (Cao *et al.*, 2008; Dai *et al.*, 2009). However, the antifungal activity of baicalein against *C. krusei* has never been reported. To evaluate the antifungal

spectrum of baicalein to other *Candida* species, in the present study, we demonstrated that baicalein possessed antifungal activity against *C. krusei* isolates and the mechanism was not through programmed cell death.

6.2 Results

6.2.1 *In vitro* susceptibility of *C. krusei* to baicalein

A summary of MIC values of baicalein on *C. krusei* is shown in Table 6.1. Baicalein exhibited potent antifungal activity against *C. krusei* isolates (MIC range = 1.35–2.70 μg/ml), comparable to that of AMB (MIC range = 0.5–1 μg/ml). Our results suggested that baicalein might exert its antifungal activity against *C. krusei* by mechanisms other than FLC resistance.

Table 6.1 The MIC value (μg/ml) of baicalein and AMB against *C. krusei* isolates.

C. krusei isolates	Baicalein	AMB
ATCC 6258	2.70	1.0
CK1	1.35	0.5
CK5	2.70	1.0
CK9	1.35	0.5
CK13	1.35	0.5
CK15	1.35	1.0
CK18	2.70	0.5
CK25	2.70	1.0

6.2.2 PAFE of baicalein on *C. krusei*

In general, PAFE was observed in baicalein-treated *C. krusei* isolates with mean value of 0.44 h (1 × MIC), 1.07 h (2 × MIC) and 1.97 h (4 × MIC). At 2 × MIC, PAFE was induced significantly in AMB while FLC only showed a marginal effect (Table 6.2).

Table 6.2 PAFE (h)* on *C. krusei* isolates after 1-h exposure to baicalein, FLC and AMB.

C. krusei isolates	Baicalein			FLC	AMB
	1 × MIC	2 × MIC	4 × MIC	2 × MIC	2 × MIC
ATCC 6258	0.49	1.20	1.70	0.35	4.90
CK1	0.25	0.69	1.83	0.25	3.95
CK5	0.52	1.03	2.13	0.28	4.39
CK9	0.26	0.65	1.35	0.33	4.65
CK13	0.61	1.83	2.02	0.23	3.58
CK15	0.35	1.25	2.10	0.19	4.25
CK18	0.55	1.16	2.49	0.41	5.15
CK25	0.45	0.77	2.11	0.38	4.57
Mean	0.44 ± 0.13	1.07 ± 0.39	1.97 ± 0.34	0.30 ± 0.08	4.43 ± 0.51

* The value is the average of three independent determinations while the mean refers to the average ± SD of the 8 *C. krusei* isolates.

6.2.3 Baicalein depolarized mitochondria in *C. krusei*

To investigate the effect of baicalein on the functions of mitochondria, we measured the change of the MMP of *C. krusei* cells after treatment with different concentrations of baicalein. Exposure of *C. krusei* cells to baicalein depolarized the MMP, as revealed by a reduction in the fluorescence intensity ratio (FL-2/FL-1) (Fig. 6.1).

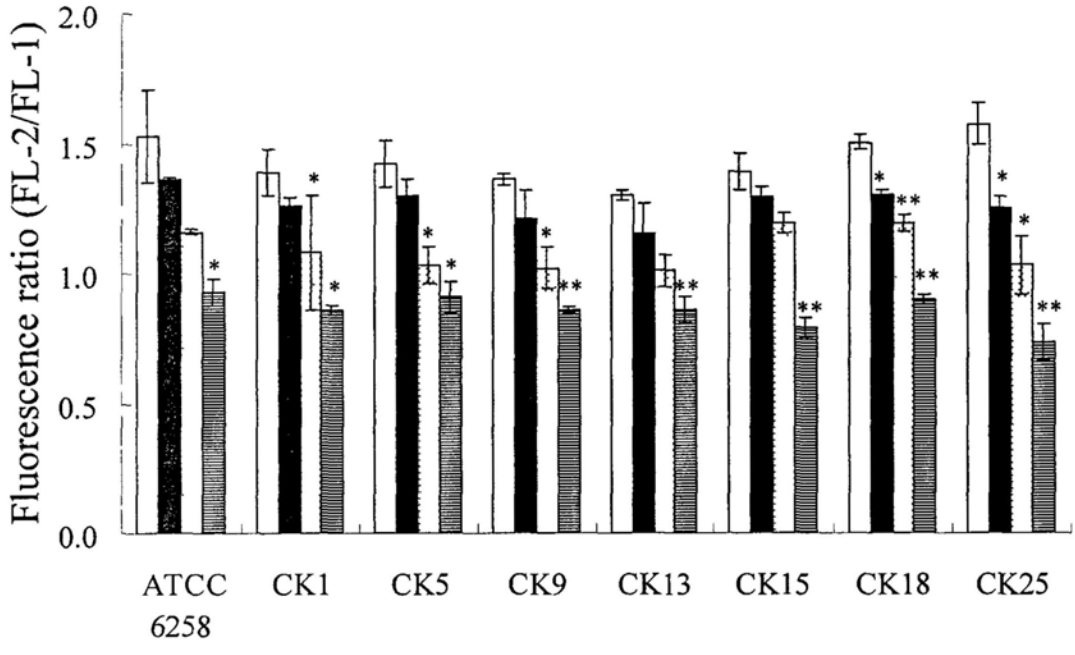


Fig. 6.1 Baicalein depolarizes MMP of *C. krusei*. The concentrations of baicalein used were 0 (□), 1×MIC (■), 2×MIC (⬚) and 4×MIC (▒). Measurements were recorded by a Beckman Coulter flow cytometer and MMP was indicated by the ratio of FL-2 (green fluorescence = 525 nm) to FL-1 (red fluorescence = 595 nm).

* $p < 0.05$, ** $p < 0.01$ when compared with the corresponding control (n = 3).

6.2.4 Baicalein did not induce ROS in *C. krusei*

The production of ROS is one of the early changes leading to apoptosis. We measured the total intracellular ROS and the results showed that there was no significant increase in ROS levels over background in all the eight *C. krusei* isolates following the 3h treatment of baicalein at three different concentrations (Fig. 6.2).

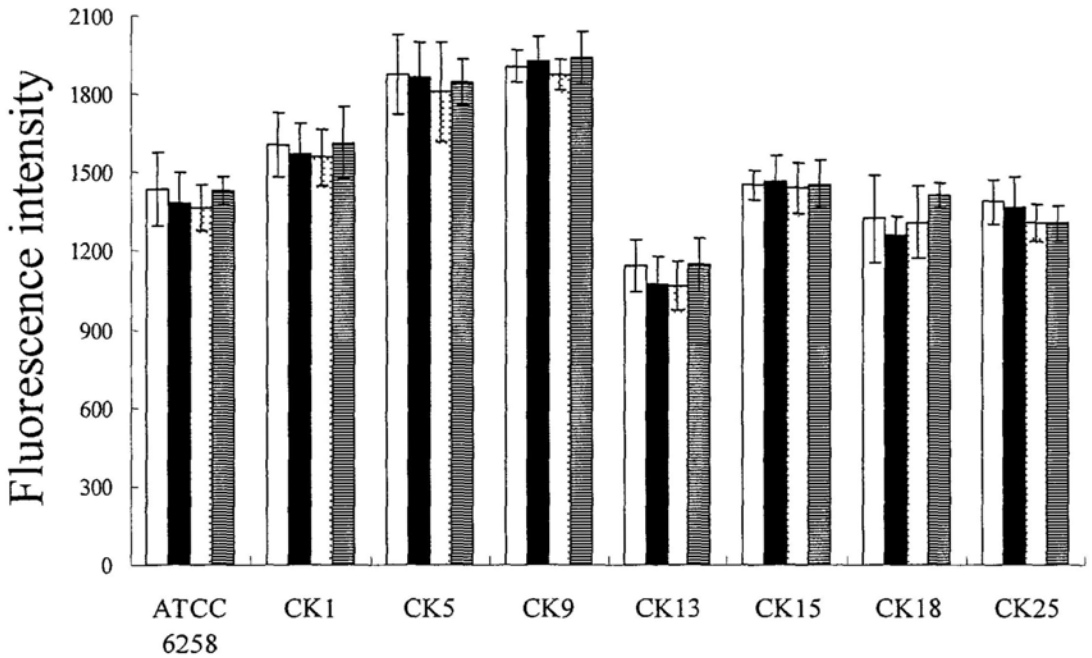


Fig. 6.2 The intracellular ROS was measured by DCFDA using a fluorometric method. The concentrations of baicalein used were 0 (□), 1×MIC (■), 2×MIC (⬚) and 4×MIC (▒).

6.2.5 Apoptosis in *Candida* cells after baicalein treatment

The increase of DNA fragmentation in the baicalein-treated *C. albicans* cells was confirmed by the results from the TUNEL assay. More than 30% cells were TUNEL positive in *C. albicans* after baicalein treatement at 10.8 μg/ml. However, only nearly 2.5% cells were positive in *C. krusei* at this concentration. While AMB, as the positive control, showed more than 40% of both *C. krusei* and *C. albicans* cells to be positive (Fig. 6.3).

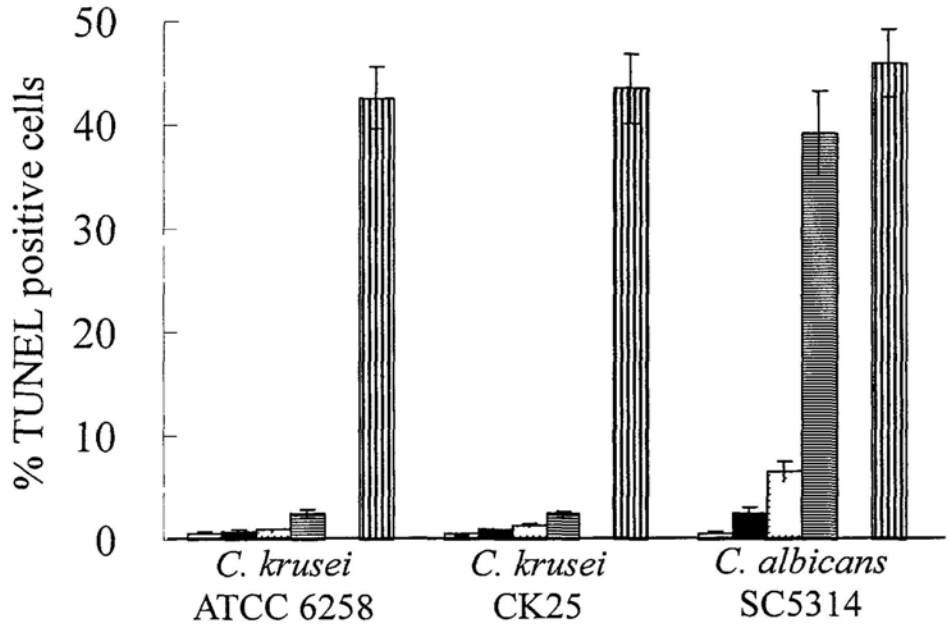


Fig. 6.3 DNA fragmentation was measured by the TUNEL assay using flow cytometry. The concentrations of baicalein used were 0 μg/ml (□), 2.7 μg/ml (■), 5.4 μg/ml (▫) and 10.8 μg/ml (▤). The concentration of AMB is 2 μg/ml (▥). The data were analyzed by WinMDI 2.9 software. Using the negative control to set a gate and record the relative values of positive cells in all groups. The data were expressed as mean ± SD (n=3).

6.2.6 Synthesis of baicalein derivatives

6.2.6.1 5,6,7 -tri-*o*-propyl-baicalein (D1)

According to the general procedure, baicalein (0.27 g, 1 mmol) was treated with propionic anhydride (1.56 g, 12 mmol) to give 5,6,7-tripropioxyflavone as a light brown crystalline solid (0.4045 g, 92%) (Fig. 6.4).

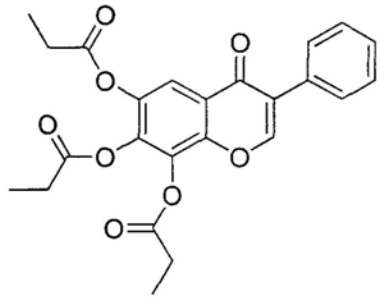


Fig. 6.4 Structure of 5,6,7-tri-*o*-propyl-baicalein

6.2.6.2 5,6,7 -tri-*o*-butyl-baicalein (D2)

According to the general procedure, baicalein (0.27 g, 1 mmol) was treated with butyric anhydride (1.896 g, 12 mmol) to give 5,6,7- tributanoyloxyflavone as a light yellow crystalline solid (0.3366 g, 75%) (Fig. 6.5).

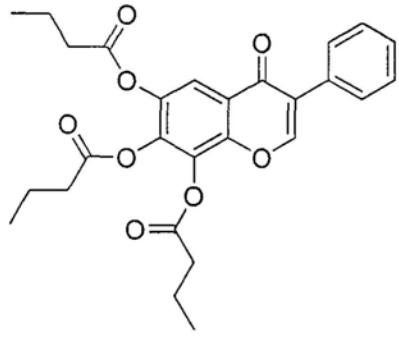


Fig. 6.5 Structure of 5,6,7-tri-*o*-butyl-baicalein

6.2.6.3 5,6,7 -tri-*o*-decanoyl-baicalein (D3)

According to the general procedure, baicalein (0.27 g, 1 mmol) was treated with decanoic anhydride (3.918 g, 12 mmol) to give 5,6,7- tridecanoyloxyflavone as a white crystalline solid (0.15 g, 21%) (Fig. 6.6).

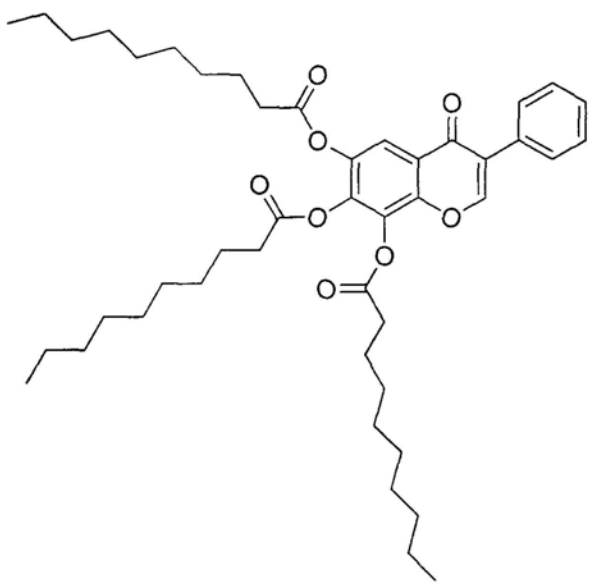


Fig. 6.6 Structure of 5,6,7-tri-*o*-decanoyl-baicalein

6.2.6.4 5,6,7 -tri-*o*-benzoyl-baicalein (D4)

According to the general procedure, baicalein (0.27 g, 1 mmol) was treated with benzoic anhydride (2.712 g, 12 mmol) to give 5,6,7- tribenzo yloxyflavone as a white crystalline solid (0.345 g, 59.7%) (Fig. 6.7).

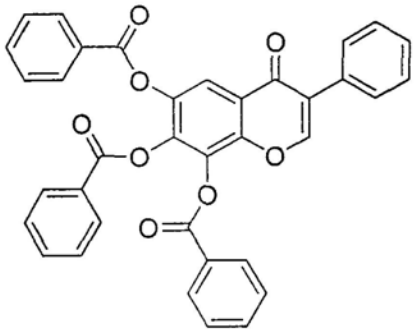


Fig. 6.7 Structure of 5,6,7-tri-*o*-benzoyl-baicalein

6.2.6.5 Antifungal activities of baicalein derivatives

Comparing to baicalein, the four derivatives showed weaker antifungal effect against several *Candida* species. The MIC of these four compounds was all larger than 80 μM, much higher than the MIC of baicalein (Table 6.3). However, comparing the antifungal activities among these four compounds, we found that the side chains did correlate with the antifungal effect. Much shorter side chain had more potent antifungal activities (Table 6.4).

Table 6.3 The antifungal activities of four baicalein derivatives against *Candida* species after 24 h of incubation.

	MIC (μM)							
	CA* ATCC 28367	CA 12	CD* MYA 646	CG* ATCC 90030	CT* ATCC 13803	CK* ATCC 6258	CP* ATCC 22014	*C. kefyr* WT-1
Baicalein	20	20	20	10	20	10	20	10
D1	> 80	> 80	> 80	> 80	> 80	> 80	> 80	> 80
D2	> 80	> 80	> 80	> 80	> 80	> 80	> 80	> 80
D3	> 80	> 80	> 80	> 80	> 80	> 80	> 80	> 80
D4	> 80	> 80	> 80	> 80	> 80	> 80	> 80	> 80

* CA: *Candida albicans*; CD: *Candida dubliensis*; CG: *Candida glabrata*; CT: *Candida tropicalis*; CK: *Candida krusei*; CP: *Candida parapsilosis*

Table 6.4 Antifungal activities of four baicalein derivatives at the final concentration of 80 μM.

	% inhibition of control							
	CA* ATCC 28367	CA 12	CD* MYA 646	CG* ATCC 90030	CT* ATCC 13803	CK* ATCC 6258	CP* ATCC 22014	*C. kefyr* WT-1
D1	27.77	48.81	61.91	64.46	65.54	64.03	72.45	66.59
D2	25.02	37.78	24.44	12.97	19.64	17.60	23.69	4.89
D3	19.14	25.14	23.74	21.27	15.72	20.39	16.63	15.26
D4	19.68	32.34	10.69	2.04	15.96	12.99	19.03	9.32

* CA: *Candida albicans*; CD: *Candida dubliensis*; CG: *Candida glabrata*; CT: *Candida tropicalis*; CK: *Candida krusei*; CP: *Candida parapsilosis*

6.3 Discussion

Baicalein is a major component of *Scutellaria baicalensis*, a traditional Chinese medicine described in the Chinese Pharmacopoeia. As one of the flavones, baicalein is present in all major land-plant lineages. The flavones-producing plant species scatter among 70 different families within the plant kindom such as the edible vegetables, fruits and seeds. Currently, they attract considerable scientific and therapeutic interest because of the assumed beneficial effect of flavone-containing food in the prevention of some human diseases. For example, epidemiology and animal studies suggested that a high dietary intake of flavones may be linked to a reduced risk of several types of cancer, coronary heart disease, chronic inflammation, and osteoporosis (Martens and Mithöfer, 2005). Baicalein is known to have antioxidant, neuroprotective, antibacterial and antiviral activities (Lin *et al.*, 2007; Chang *et al.*, 2007a; Chang *et al.*, 2007c; Wu *et al.*, 2001). Recently, Dai *et al* also reported the antifungal activity of baicalein against *C. albicans* (Huang *et al.*, 2008;

Cao *et al.*, 2008; Dai *et al.*, 2009). Our studies highlighted the extension of antifungal spectrum of baicalein to the FLC resistant *C. krusei*, which responded to Dai's finding and confirmed the antifungal activity of baicalein. However, there are some differences in the antifungal mechanism between *C. albicans* and *C. krusei*.

Baicalein can depolarize the MMP in both *C. albicans* (Dai *et al.*, 2009) and *C. krusei*, which means that the antifungal activity of baicalein is mitochondria-dependent. Onset of mitochondrial-dependent apoptotic pathway leads to translocation of cytochrome *c* to cytosol and nucleus, release of apoptotic inducing factor, apoptosome formation and step-wise activation of caspase-3 and -9 (Ramsdale, 2008).

In the present study, we found that the total cellular level of ROS in *C. krusei* was not changed significantly after the treatment of baicalein, which was in disagreement with the results of previous study in *C. albicans* (Dai *et al.*, 2009). As a consequence, we also did not detect any DNA fragmentation by TUNEL assay in *C. krusei*, however, we found baicalein did work on *C. albicans* at the same concentration and incubation time. These evidence showed that *C. krusei* and *C. albicans* had different susceptibility and response to baicalein. A recent study on the antileishmanial activity of sertraline suggested a linkage between drug-induced apoptosis and inhibition of phospholipase enzymes (Palit and Ali, 2008). Baicalein can inhibit intracellular concentration of Ca^{2+} elevation by reducing phospholipase C activity (Kyo *et al.*, 1998), which may interpret the difference of response to baicalein as there might be no apoptotic target for baicalein in *C. krusei* (Gokce *et al.*, 2007).

Rational drug design does not always acquire effective antimicrobial compounds. To yield more potent antifungal agents and investigate the relationship between structure and antifungal activities of baicalein, we synthesized four derivatives by acylation of the three hydroxyl groups of baicalein. After comparing the antifungal activities, it was clear that the free hydroxyl groups were indispensable for baicalein, which might be due to its antioxidative activity by ROS scavenging. Recent pharmacological studies of baicalein have demonstrated the antioxidative, anti-inflammatory and neuroprotective effects (Shieh *et al.* 2000; van Leyen *et al.* 2008; Yang *et al.* 2000). One of its antioxidative mechanisms is ROS scavenging because of the three free phenolic hydroxyl groups. These groups are capable of transfering electron free radicals and interfere with the oxidation of lipids or other molecules by the donation of hydrogen atom (Min, 2009). There is also report claiming that the number and position of hydroxyl group in the ring of baicalein were associated with its anti-tumor activity (Chang *et al.* 2007b). The role of the number and position of hydroxyl group in the ring of baicalein need to be further studied.

In conclusion, the present study provided evidence for the antifungal activity of baicalein against intrinsically FLC resistant *C. krusei*. Although baicalein-induced toxicity shares the apoptotic feature of depolarization of MMP, the present data show that none of the other apoptotic markers, including ROS production and DNA fragmentation, accompany baicalein-induced cell death. We can conclude that baicalein-induced cell death does not occur through apoptosis and there would be a possible linkage of baicalein treatment to triggering of mitochondria-dependent pathway in *C. krusei* considering the observation of MMP depolarization. Chemical-induced cell death in fungi is intricate and further studies on the effects of

baicalein in *C. krusei* are needed to investigate the molecular mechanism and to harness its antifungal potential.

Chapter 7
Conclusion

After screening a compound library with 400 members against a panel of 20 *Candida* strains, five compounds (1.25% of the library) exhibited potent effect in inhibiting the fungal growth and they were regarded as potential antifungal agents for further studies. Nine compounds (2.25% of the library) showed moderate effect in inhibiting the fungal growth. The remaining compounds exhibited no antifungal activities. Among the five compounds, two of them, named anisomycin and cycloheximide, have been reported with antifungal activities. So we studied the other three pure compounds: metergoline, purpurin and baicalein.

Although metergoline and purpurin were not structure-new compounds, they have never been reported with antifungal activities before. Baicalein has been established its antifungal activities against *C. albicans*, but it was the first time to report that baicalein inhibited the growth of *C. krusei*, a FLC-resistant strain. All these three compounds showed a broad spectrum of antifungal activities against *Candida* species, however, they demonstrated that they were more potent against *C. krusei*.

All these three compounds showed basic antifungal characteristics, while their antifungal mechanisms were different. If comparing the antifungal potency against *C. krusei*, the MIC value of purpurin was the lowest to the other two compounds (5 μM *vs.* 10 μM). Even more interesting, purpurin was an antifungal agent with the effect of inhibiting the efflux pump, which facilitates it to be used for drug resistant infection. The most promising of metergoline was that it was a fungicidal agent,

which had significant meaning in clinical treatment.

Interestingly, established antidepressants, including the selective inhibitors of serotonin reuptake such as sertraline, have been reported to have a significant potential of induing adverse drug reactions. Concentrations of these drugs increased significantly in the brain of *mdr* (-/-) knockout mice after single dose administration, suggesting that these antidepressants are P-glycoprotein substrates and might also have potential inhibitory effect on the P-glycoprotein (Weiss *et al.*, 2003). Therefore, as a serotonin receptor antagonist, metergoline may also have the ability to inhibit the efflux of R6G from *Candida* species. Provided that further experiments can confirm its inhibitory ability on the efflux pump, we may acquire one more antifungal compound with the ability of reversing multidrug resistance, just like purpurin.

All these three compounds could depolarize mitochondrial membrane potential, while only metergoline was tested for the induction of programmed cell death in *C. krusei*, so the loss of mitochondrial membrane potential could not be used as a direct evidence to deduce the process of apoptosis. Detecting other indicators following the loss of mitochondrial membrane potential will be necessary to confirm the occurrence of apoptosis in cell.

Metergoline could induce apoptosis in *C. krusei*, which was ROS-dependent and might be the fungicidal mechanism. The antifungal activity of baicalein was not associated with apoptosis in *C. krusei*, although it has been confirmed that baicalein could induce apoptosis in *C. albicans*. Flavones are known to be potent and selective inhibitors of proliferation, as well as inducers of differentiation and apoptosis in

colon cancer cells. This high selectivity for the induction of apoptosis and growth inhibition was only observed in the transformed but not in primary non-transformed colonocytes (Wenzel *et al.*, 2000). The underlying mechanism of this selective activity is probably the enhanced uptake of monocarboxylates such as lactate into mitochondria most likely by an allosteric activation of monocarboxylate transporter-1 (Martens and Mithöfer, 2005). In this study, baicalein also showed selectivity of the action on *C. krusei* and *C. albicans*. However, the mechanisms of different compounds on the same strain and the same compound on different strains need to be further studied.

As these three compounds can intensify the antifungal potency of AMB and FLC, they can be used as an adjunct to treat some facial *Candida* infection, lowering down the toxicity of AMB or reducing the incidence of emergence of FLC resistance.

Caspases belong to a family of proteins that are one of the main executors of the apoptotic process. They belong to the group of enzymes known as cysteine protease and exist within the cell as inactive pro-forms or zymogens. These zymogens can be cleaved to form active enzymes following the induction of apoptosis.

Induction of apoptosis via death receptors typically results in the activation of an initiator caspase such as caspase 8 or caspase 10. These caspases can then activate other caspases in a cascade. This cascade eventually leads to the activation of the effector caspases, such as caspase 3 and caspase 6. These caspases are responsible for the cleavage of the key cellular proteins, such as cytoskeletal proteins, that leads to the typical morphological changes observed in cells undergoing apoptosis.

Release of cytochrome C from mitochondria can lead to the activation of caspase 9, and then of caspase 3. This effect is mediated through the formation of an apoptosome, a multi-protein complex consisting of cytochrome C, Apaf-1, pro-caspase 9 and ATP. The exact apoptotic mechanism induced by metergoline, especially the role of caspase and cytochrome c is important in further studies to explore the clinical potential of this drug.

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Appendix Ⅰ Screening data of the antifungal agents in the compound library

Compound No	*C dubliensis* MYA646	*C glabrata* ATCC 90030	*C glabrata* WT-5	*C glabrata* 54640	*C krusei* ATCC 6258	*C krusei* CK25	*C tropicalis* ATCC 13803	*C tropicalis* 90/1362	*C tropicalis* 58660	*C parapsilosis* ATCC 22014	*C parapsilosis* 90/2224	*C albicans* SAP 1-3	*C albicans* SAP 4-6	*C albicans* ATCC 18804	*C albicans* ATCC 28367	*C albicans* 71929	*C albicans* 12	*C albicans* 194	*C kefyr* WT-1	*C guilliermondii* 192006
1	3	4	3	4	4	2	2	3	4	2	4	4	4	3	3	4	3	4	3	3
2	3	3	3	4	4	2	3	4	4	3	4	3	4	3	2	4	2	4	3	2
3	3	3	3	3	4	1	3	4	4	4	4	4	3	4	2	4	4	4	4	2
4	4	4	2	3	4	2	2	4	4	3	4	4	4	4	3	4	4	3	3	1
5	3	2	2	4	4	4	2	3	4	4	4	4	4	4	3	4	2	3	3	3
6	3	2	3	4	4	3	2	3	4	4	4	3	4	3	2	3	3	3	3	2
7	2	3	3	3	3	3	3	3	2	4	3	3	4	2	3	3	3	2	4	3
8	4	4	3	3	4	2	4	3	2	4	4	4	4	3	3	3	2	3	3	3
9	2	3	3	4	4	2	2	2	3	3	4	3	3	4	2	4	3	3	4	2
10	3	2	3	4	4	3	2	3	4	4	3	3	4	3	3	3	2	3	3	2
11	3	3	2	3	3	2	3	2	3	4	3	4	4	3	3	3	2	2	3	3
12	4	4	3	4	4	2	4	3	2	2	4	3	4	4	3	4	3	3	2	3
13	3	3	3	4	4	4	2	3	4	4	3	3	3	4	2	4	4	3	3	2
14	3	2	3	4	4	3	2	2	4	3	3	2	4	4	3	3	4	2	3	3
15	3	3	3	3	4	3	2	3	2	2	3	3	4	4	2	4	3	3	3	4
16	4	4	3	4	4	3	3	2	2	3	4	4	3	4	2	4	3	3	2	2
17	3	3	3	4	4	4	2	4	4	4	3	4	3	3	3	3	4	4	4	3
18	3	2	3	4	3	3	2	3	4	3	2	4	4	3	4	2	4	3	2	3
19	2	3	2	4	3	2	3	2	3	2	2	4	4	3	3	4	3	3	3	3
20	4	4	4	4	4	2	3	3	3	2	4	3	3	4	3	4	4	4	3	4

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Compound No	*C dubliensis* MYA646	*C glabrata* ATCC 90030	*C glabrata* WT-5	*C glabrata* 54640	*C krusei* ATCC 6258	*C krusei* CK25	*C tropicalis* ATCC 13803	*C tropicalis* 90/1362	*C tropicalis* 58660	*C parapsilosis* ATCC 22014	*C parapsilosis* 90/2224	*C albicans* SAP 1-3	*C albicans* SAP 4-6	*C albicans* ATCC 18804	*C albicans* ATCC 28367	*C albicans* 71929	*C albicans* 12	*C albicans* 194	*C kefyr* WT-1	*C guilliermondii* 192006
21	3	3	4	4	4	4	2	4	4	4	3	3	4	4	4	4	4	4	3	3
22	3	3	3	4	3	3	3	3	3	4	2	3	4	3	4	3	4	3	2	2
23	4	2	3	4	4	2	3	2	4	2	4	3	3	3	3	4	4	4	3	3
24	4	4	3	4	4	2	3	4	4	3	4	4	4	4	2	4	4	4	3	3
25	3	2	3	4	4	4	3	3	3	4	3	4	4	3	2	3	4	4	3	4
26	3	2	3	2	2	4	3	2	3	4	2	2	2	3	4	2	3	2	2	2
27	4	3	3	3	4	4	2	3	4	3	4	4	3	3	3	4	4	3	3	3
28	4	4	3	4	4	3	3	4	3	3	4	3	4	4	3	4	4	4	3	4
29	4	3	4	4	4	3	3	4	4	4	3	3	4	4	3	3	4	4	3	3
30	3	2	3	4	3	3	3	3	3	4	2	1	3	3	2	2	4	3	2	2
31	3	2	3	4	4	4	3	4	4	2	2	3	4	3	4	4	3	3	3	3
32	4	3	3	4	4	4	4	4	3	4	4	4	3	4	4	4	4	4	3	4
33	4	3	4	4	4	2	4	4	3	4	3	4	3	4	3	4	4	4	3	3
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35	3	4	3	3	4	3	4	4	3	3	2	3	4	4	4	3	4	3	2	3
36	4	4	2	4	4	4	4	3	3	3	4	4	4	3	4	4	4	3	3	4
37	4	4	4	4	3	2	4	4	3	4	2	4	4	4	2	4	4	4	3	3
38	3	4	2	4	3	3	4	4	4	4	2	2	2	4	3	2	4	4	3	2
39	3	4	4	4	4	4	4	4	3	3	3	3	4	3	3	3	3	4	2	3
40	4	4	3	4	4	4	3	3	4	3	4	4	3	4	4	3	3	4	2	2

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Compound No	*C dubliensis* MYA646	*C glabrata* ATCC 90030	*C glabrata* WT-5	*C glabrata* 54640	*C krusei* ATCC 6258	*C krusei* CK25	*C tropicalis* ATCC 13803	*C tropicalis* 90/1362	*C tropicalis* 58660	*C parapsilosis* ATCC 22014	*C parapsilosis* 90/2224	*C albicans* SAP 1-3	*C albicans* SAP 4-6	*C albicans* ATCC 18804	*C albicans* ATCC 28367	*C albicans* 71929	*C albicans* 12	*C albicans* 194	*C kefyr* WT-1	*C guilliermondii* 192006
41	4	4	4	4	4	3	4	4	3	4	3	4	4	4	2	3	4	4	4	3
42	4	4	3	4	3	3	4	4	3	4	2	2	2	3	2	2	4	4	3	2
43	2	4	4	4	4	3	3	4	3	4	3	3	4	4	3	3	4	4	3	3
44	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	3	4	3	3
45	3	4	4	3	4	3	4	4	4	4	3	4	4	2	4	4	4	3	3	3
46	3	3	3	3	3	3	2	3	4	4	4	4	4	4	4	4	2	4	2	3
47	3	2	4	3	3	3	2	4	4	4	4	3	4	3	2	4	3	4	3	3
48	3	2	3	3	4	2	2	4	3	3	4	3	4	3	3	3	2	4	3	3
49	2	3	3	3	4	2	2	4	3	4	4	4	4	3	4	3	2	4	2	4
50	3	3	2	2	4	4	2	3	4	4	3	3	4	4	3	4	3	3	3	4
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52	4	2	2	3	4	3	3	3	2	4	3	3	4	3	2	3	4	4	3	3
53	***4***	***3***	***4***	***4***	***4***	***2***	***2***	***3***	***2***	***3***	***4***	***4***	***4***	***3***	***3***	***4***	***3***	***3***	***2***	***4***
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55	3	2	3	3	4	3	2	3	3	4	2	2	2	4	2	2	2	3	3	2
56	3	3	3	4	4	3	3	3	2	4	4	4	4	3	2	3	3	4	3	3
57	3	3	4	4	4	2	2	4	3	3	4	3	4	4	4	4	3	3	3	4
58	3	4	3	3	3	2	3	4	4	4	3	4	4	4	3	4	4	4	4	4
59	3	3	3	3	4	2	3	4	3	3	2	2	2	3	2	2	3	3	4	3
60	4	2	2	4	4	2	3	4	2	4	3	4	4	4	2	3	4	3	3	4

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Compound No	*C dubliensis* MYA646	*C glabrata* ATCC 90030	*C glabrata* WT-5	*C glabrata* 54640	*C krusei* ATCC 6258	*C krusei* CK25	*C tropicalis* ATCC 13803	*C tropicalis* 90/1362	*C tropicalis* 58660	*C parapsilosis* ATCC 22014	*C parapsilosis* 90/2224	*C albicans* SAP 1-3	*C albicans* SAP 4-6	*C albicans* ATCC 18804	*C albicans* ATCC 28367	*C albicans* 71929	*C albicans* 12	*C albicans* 194	*C kefyr* WT-1	*C guilliermondii* 192006
61	3	3	3	4	4	3	2	3	2	4	4	3	3	3	3	4	2	4	3	3
62	2	4	3	2	4	3	3	2	4	4	3	4	3	4	2	3	4	4	4	3
63	1	3	2	2	3	2	4	2	3	4	2	2	2	3	3	2	4	3	3	4
64	3	3	2	4	4	3	3	3	2	4	3	4	4	2	4	3	4	3	3	4
65	2	4	3	4	4	4	3	4	2	4	4	4	3	3	3	4	3	4	3	4
66	3	4	3	3	4	2	3	3	4	4	4	4	3	4	3	3	4	4	3	3
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70	3	3	3	3	3	3	3	3	4	4	3	4	4	4	3	4	4	4	3	3
71	2	3	3	2	4	3	4	2	3	4	2	3	3	3	3	1	4	3	4	2
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74	3	4	3	4	4	3	4	4	4	3	3	4	4	4	3	4	4	4	4	3
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76	3	2	3	3	4	4	3	3	2	3	3	4	3	4	4	3	4	4	3	3
77	2	3	3	4	4	4	3	4	2	4	4	4	4	3	3	4	4	4	2	4
78	3	4	3	4	3	3	3	4	4	3	3	4	4	4	3	3	4	4	4	3
79	3	3	3	4	3	3	3	4	3	3	2	2	3	4	3	2	4	4	4	2
80	3	2	3	3	4	4	4	3	2	2	3	2	3	4	3	2	3	4	3	3

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Compound No	*C dubliensis* MYA646	*C glabrata* ATCC 90030	*C glabrata* WT-5	*C glabrata* 54640	*C krusei* ATCC 6258	*C krusei* CK25	*C tropicalis* ATCC 13803	*C tropicalis* 90/1362	*C tropicalis* 58660	*C parapsilosis* ATCC 22014	*C parapsilosis* 90/2224	*C albicans* SAP 1-3	*C albicans* SAP 4-6	*C albicans* ATCC 18804	*C albicans* ATCC 28367	*C albicans* 71929	*C albicans* 12	*C albicans* 194	*C kefyr* WT-1	*C guilliermondii* 192006
81	2	3	4	4	3	4	3	4	2	4	4	3	4	3	4	3	3	4	2	4
82	3	4	3	4	3	3	3	4	4	4	3	4	3	4	2	3	4	4	3	3
83	4	3	3	4	3	2	3	3	3	3	2	2	3	4	3	2	4	4	2	3
84	2	2	2	4	3	3	3	3	2	3	3	3	4	4	2	3	3	3	2	3
85	3	3	4	4	3	4	4	4	2	4	4	3	3	4	4	3	3	4	3	4
86	3	4	4	4	4	4	3	4	4	4	2	4	4	4	3	3	4	4	3	3
87	4	4	3	4	3	3	3	3	2	4	2	2	2	4	2	2	4	4	2	3
88	2	4	3	4	4	3	4	4	2	4	3	3	4	4	3	3	3	4	2	4
89	3	3	4	4	3	4	3	4	3	4	4	4	4	4	4	3	2	3	2	3
90	4	3	4	4	4	4	3	4	4	4	4	4	4	4	4	4	4	4	4	4
91	4	4	4	3	3	4	3	3	4	3	4	4	4	3	4	4	2	3	4	4
92	3	4	3	3	4	2	2	2	3	4	4	3	4	2	2	4	4	2	3	4
93	***2***	***0***	***1***	***1***	***0***	***0***	***1***	***2***	***1***	***0***	***2***	***1***	***0***	***2***	***0***	***1***	***2***	***0***	***0***	***2***
94	2	4	3	4	4	3	2	3	3	3	4	4	4	3	4	4	4	3	2	4
95	2	4	3	2	3	4	2	2	2	3	4	4	4	3	4	4	3	4	3	3
96	2	3	3	2	3	3	3	1	2	3	4	3	4	3	2	3	4	4	2	2
97	3	4	4	2	4	3	3	3	2	4	4	3	3	3	3	2	4	3	3	4
98	2	4	4	4	4	4	2	4	3	4	4	4	4	1	4	3	4	4	3	4
99	3	4	4	2	2	3	4	2	2	4	4	4	4	2	4	4	3	4	3	3
100	***2***	***4***	***3***	***1***	***3***	***3***	***3***	***1***	***2***	***3***	***3***	***1***	***2***	***3***	***2***	***2***	***4***	***2***	***2***	***2***

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Compound No.	*C dubliensis* MYA646	*C glabrata* ATCC 90030	*C glabrata* WT-5	*C glabrata* 54640	*C krusei* ATCC 6258	*C krusei* CK25	*C tropicalis* ATCC 13803	*C tropicalis* 90/1362	*C tropicalis* 58660	*C parapsilosis* ATCC 22014	*C parapsilosis* 90/2224	*C albicans* SAP 1-3	*C albicans* SAP 4-6	*C albicans* ATCC 18804	*C albicans* ATCC 28367	*C albicans* 71929	*C albicans* 12	*C albicans* 194	*C kefyr* WT-1	*C guilliermondii* 192006
101	3	4	3	2	3	3	2	2	2	3	3	2	3	3	2	2	4	4	2	4
102	2	4	4	3	4	4	2	4	3	4	4	3	4	2	3	3	4	4	3	4
103	4	4	4	3	3	3	3	3	4	4	3	4	3	2	4	2	3	4	4	3
104	3	3	3	2	3	2	4	2	4	3	3	3	4	3	2	2	4	3	2	2
105	3	3	3	3	4	3	3	1	2	3	4	2	4	2	2	2	3	4	2	4
106	4	4	4	4	4	3	3	4	2	4	4	3	4	2	3	3	4	4	3	4
107	4	4	4	4	3	3	3	3	4	4	3	4	3	2	3	2	4	3	3	3
108	3	4	3	3	2	2	3	2	4	3	2	2	2	3	3	2	4	4	2	4
109	4	3	3	3	4	3	3	2	3	3	3	2	3	2	3	2	3	4	2	3
110	3	4	4	4	4	4	3	3	3	4	4	4	3	3	4	3	4	4	3	4
111	3	4	4	3	3	3	2	3	3	4	4	4	3	3	4	2	4	3	4	4
112	3	3	4	2	2	2	3	2	3	3	2	3	3	2	4	3	4	2	3	4
113	4	3	4	3	3	3	3	2	2	3	3	3	3	2	4	2	4	3	3	4
114	4	4	4	4	4	4	4	2	2	4	4	4	3	3	3	4	4	4	3	4
115	4	4	4	2	4	3	4	3	2	3	4	4	4	3	3	3	4	4	3	4
116	3	3	4	2	4	2	3	2	3	2	2	2	3	3	4	2	4	2	2	2
117	3	3	4	3	4	3	3	2	3	2	3	2	2	2	3	2	4	3	3	3
118	3	4	4	4	4	3	2	2	3	4	3	4	3	3	3	3	4	3	2	4
119	2	4	4	2	2	2	2	3	2	3	2	3	3	4	2	3	4	3	3	3
120	3	3	4	2	3	3	3	2	3	3	2	2	3	3	3	2	4	4	2	2

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Compound No.	*C dubliensis* MYA646	*C glabrata* ATCC 90030	*C glabrata* WT-5	*C glabrata* 54640	*C krusei* ATCC 6258	*C krusei* CK25	*C tropicalis* ATCC 13803	*C tropicalis* 90/1362	*C tropicalis* 58660	*C parapsilosis* ATCC 22014	*C parapsilosis* 90/2224	*C albicans* SAP 1-3	*C albicans* SAP 4-6	*C albicans* ATCC 18804	*C albicans* ATCC 28367	*C albicans* 71929	*C albicans* 12	*C albicans* 194	*C kefyr* WT-1	*C guilliermondii* 192006
121	3	3	4	4	4	3	4	2	4	2	3	2	3	2	4	2	4	4	4	4
122	3	4	4	4	4	4	4	2	3	4	4	3	4	3	3	3	4	4	3	4
123	3	4	4	2	3	3	3	3	2	2	4	4	4	4	2	2	4	4	3	3
124	3	3	4	1	2	3	3	2	3	3	2	2	4	3	3	1	4	4	2	3
125	2	3	4	2	4	3	4	2	4	2	3	2	3	3	3	2	3	4	3	4
126	3	4	4	2	4	4	3	3	3	4	4	3	3	2	3	3	4	4	3	4
127	3	3	3	2	4	3	3	3	2	3	2	4	4	3	2	3	3	4	2	4
128	3	3	3	2	2	3	3	3	2	3	2	2	3	3	3	1	3	4	3	2
129	3	4	3	2	3	3	3	2	3	2	3	3	4	3	3	2	3	4	4	3
130	3	4	4	2	4	4	3	3	4	3	3	4	4	3	4	3	4	4	4	4
131	4	4	4	3	3	4	3	3	2	2	3	4	3	4	3	3	4	4	3	4
132	3	4	3	3	2	3	3	2	2	4	2	2	4	4	3	2	3	4	3	4
133	3	4	4	2	3	4	4	2	3	3	3	3	4	3	3	3	4	4	3	3
134	3	4	4	4	4	4	4	3	4	3	4	4	4	4	4	4	4	4	3	4
135	4	2	3	4	4	3	3	4	3	3	3	4	4	3	3	3	4	4	3	3
136	4	4	4	3	3	3	4	4	4	3	4	3	4	2	2	4	4	4	4	3
137	4	4	2	3	3	2	4	3	4	4	4	4	4	3	3	4	4	4	4	3
138	4	4	2	3	4	3	4	2	3	4	4	3	4	3	3	4	4	4	3	2
139	4	4	2	4	4	2	4	4	3	3	4	4	4	2	3	4	4	4	4	4
140	4	4	1	2	4	4	4	3	4	4	4	3	4	4	2	4	4	3	4	2

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Compound No.	*C dubliensis* MYA646	*C glabrata* ATCC 90030	*C glabrata* WT-5	*C glabrata* 54640	*C krusei* ATCC 6258	*C krusei* CK25	*C tropicalis* ATCC 13803	*C tropicalis* 90/1362	*C tropicalis* 58660	*C parapsilosis* ATCC 22014	*C parapsilosis* 90/2224	*C albicans* SAP 1-3	*C albicans* SAP 4-6	*C albicans* ATCC 18804	*C albicans* ATCC 28367	*C albicans* 71929	*C albicans* 12	*C albicans* 194	*C kefyr* WT-1	*C guilliermondii* 192006
141	3	3	2	2	3	3	4	2	4	4	4	4	4	4	2	3	4	4	3	3
142	4	3	1	2	3	2	4	2	2	4	4	3	4	3	4	3	4	4	2	4
143	4	3	2	4	3	1	4	3	3	3	3	4	4	3	3	3	4	2	2	4
144	3	4	2	3	4	4	4	3	4	4	4	4	4	4	2	4	4	3	3	3
145	3	3	2	2	4	3	4	2	3	3	2	4	4	3	1	4	4	4	2	2
146	4	3	4	2	3	3	4	2	2	4	3	3	4	2	3	3	4	4	3	3
147	3	3	2	3	3	3	4	2	3	4	4	3	3	3	2	4	4	4	4	4
148	3	4	4	3	2	3	4	3	4	4	4	4	3	3	3	4	4	3	3	3
149	3	3	4	2	3	2	4	2	2	3	3	4	3	3	1	3	4	3	2	3
150	4	4	4	2	2	3	4	2	3	4	2	2	2	2	2	2	4	3	3	3
151	3	3	2	3	3	2	4	2	3	4	3	3	3	4	3	4	4	2	4	4
152	4	4	4	3	4	2	4	3	4	4	4	4	3	3	2	4	4	4	3	4
153	3	3	3	2	3	2	4	2	3	4	4	4	3	3	2	4	4	3	3	3
154	4	3	4	2	2	2	4	1	3	4	2	4	3	3	3	3	4	2	2	2
155	4	4	4	4	4	4	4	3	3	4	3	4	3	3	4	4	4	4	3	4
156	4	4	4	3	3	3	3	3	4	3	4	3	4	3	2	3	4	3	3	3
157	3	4	3	2	4	2	4	2	4	3	4	3	4	3	2	3	4	3	3	2
158	3	4	4	2	3	3	4	2	3	4	3	4	3	3	3	3	4	3	2	4
159	4	4	4	3	4	4	4	3	2	4	3	4	4	3	4	4	4	4	4	3
160	4	4	4	4	3	3	4	3	4	3	4	3	4	3	2	4	4	4	3	3

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Compound No	*C dubliensis* MYA646	*C glabrata* ATCC 90030	*C glabrata* WT-5	*C glabrata* 54640	*C krusei* ATCC 6258	*C krusei* CK25	*C tropicalis* ATCC 13803	*C tropicalis* 90/1362	*C tropicalis* 58660	*C parapsilosis* ATCC 22014	*C parapsilosis* 90/2224	*C albicans* SAP 1-3	*C albicans* SAP 4-6	*C albicans* ATCC 18804	*C albicans* ATCC 28367	*C albicans* 71929	*C albicans* 12	*C albicans* 194	*C kefyr* WT-1	*C guilliermondii* 192006
161	3	4	3	2	3	3	4	2	4	2	3	2	4	2	3	2	4	3	3	2
162	4	4	3	2	2	3	4	2	3	3	2	3	4	3	4	3	4	2	2	3
163	4	4	4	3	4	4	4	3	3	4	3	4	4	3	4	4	4	4	3	3
164	4	3	3	3	3	2	4	3	3	4	4	4	3	3	3	4	4	4	3	4
165	4	4	3	2	3	3	4	2	4	2	4	3	4	2	3	2	4	4	3	2
166	4	4	3	1	3	4	4	2	3	3	2	2	4	3	4	2	4	4	2	3
167	4	4	4	3	4	4	4	3	3	4	3	4	4	3	3	4	4	4	4	3
168	4	4	4	3	3	2	4	3	3	3	4	4	4	3	2	3	4	4	3	3
169	4	3	3	2	2	3	4	2	4	2	2	2	2	3	4	2	4	4	3	2
170	4	3	3	1	3	4	4	2	3	3	3	2	2	3	4	2	4	4	2	3
171	Insoluble in DMSO																			
172	4	4	4	3	3	2	3	3	2	3	3	3	4		2	3	4	4	4	4
173	2	3	3	2	1	3	3	2	3	1	2	3	4	3	4	3	2	4	2	3
174	4	3	3	2	4	4	4	2	2	4	4	3	4	3	3	2	4	3	3	3
175	4	4	3	3	4	4	4	3	4	4	4	4	3	3	3	4	4	4	3	3
176	3	4	4	4	3	3	4	3	3	4	3	3	3	4	3	3	4	4	4	4
177	3	3	3	3	4	3	4	2	2	4	4	3	4	3	4	4	3	4	3	4
178	***2***	***1***	***2***	***0***	***0***	***1***	***3***	***1***	***0***	***1***	***0***	***1***	***1***	***4***	***1***	***1***	***3***	***1***	***1***	***0***
179	4	3	4	4	3	4	4	3	4	3	4	4	3	4	3	3	4	4	4	4
180	2	4	3	4	4	3	4	3	4	4	4	4	4	4	3	4	3	4	4	4

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Compound No	*C dubliensis* MYA646	*C glabrata* ATCC 90030	*C glabrata* WT-5	*C glabrata* 54640	*C krusei* ATCC 6258	*C krusei* CK25	*C tropicalis* ATCC 13803	*C tropicalis* 90/1362	*C tropicalis* 58660	*C parapsilosis* ATCC 22014	*C parapsilosis* 90/2224	*C albicans* SAP 1-3	*C albicans* SAP 4-6	*C albicans* ATCC 18804	*C albicans* ATCC 28367	*C albicans* 71929	*C albicans* 12	*C albicans* 194	*C kefyr* WT-1	*C guilliermondii* 192006
181	4	4	4	4	4	3	4	4	2	4	4	4	4	2	3	4	4	4	3	4
182	4	4	3	2	3	2	3	2	2	3	4	4	4	2	2	4	4	4	4	3
183	4	4	4	3	4	3	4	2	3	4	3	3	4	3	4	4	4	4	4	3
184	4	4	3	3	4	2	4	2	3	3	3	4	4	4	2	3	4	4	2	2
185	4	4	4	3	4	2	4	2	2	4	3	4	4	3	3	3	4	4	4	4
186	3	3	3	2	4	2	4	2	2	4	2	4	3	3	3	3	4	4	3	3
187	3	4	3	2	4	3	3	2	2	3	3	4	4	3	4	2	4	4	3	3
188	4	4	4	3	3	3	4	3	2	3	3	4	4	3	2	3	4	3	3	2
189	4	4	4	3	4	3	3	4	3	4	3	3	3	3	3	3	4	4	3	4
190	4	3	4	3	3	2	4	3	3	2	2	3	3	3	2	3	4	4	3	2
191	3	4	3	2	4	2	3	2	2	3	3	4	4	4	4	3	4	4	2	3
192	4	4	3	3	3	3	4	3	2	4	3	4	4	4	2	4	4	4	3	2
193	4	4	4	3	4	2	4	3	3	4	3	3	4	2	2	4	4	4	4	4
194	3	2	4	2	4	2	4	2	2	2	2	3	4	2	3	3	4	4	3	3
195	4	3	4	2	3	2	3	1	2	3	2	3	4	3	4	2	4	4	2	3
196	4	4	3	4	3	3	4	3	3	4	3	3	3	3	3	4	4	4	4	2
197	4	4	3	4	2	4	4	4	3	4	4	4	3	2	2	4	4	4	3	4
198	3	2	3	2	3	2	4	2	3	3	4	4	4	3	3	3	4	3	3	3
199	3	3	4	2	3	2	4	2	2	3	3	2	3	3	3	2	4	4	2	3
200	4	4	4	3	4	4	4	3	2	4	3	3	3	3	2	4	4	4	3	2

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Compound No	*C dubliensis* MYA646	*C glabrata* ATCC 90030	*C glabrata* WT-5	*C glabrata* 54640	*C krusei* ATCC 6258	*C krusei* CK25	*C tropicalis* ATCC 13803	*C tropicalis* 90/1362	*C tropicalis* 58660	*C parapsilosis* ATCC 22014	*C parapsilosis* 90/2224	*C albicans* SAP 1-3	*C albicans* SAP 4-6	*C albicans* ATCC 18804	*C albicans* ATCC 28367	*C albicans* 71929	*C albicans* 12	*C albicans* 194	*C kefyr* WT-1	*C guilliermondii* 192006
201	4	4	4	3	2	4	4	3	3	4	3	4	3	2	3	4	4	4	3	4
202	3	3	4	2	3	2	4	2	2	2	3	4	4	3	3	4	4	3	4	3
203	3	4	4	3	4	2	4	3	2	4	4	3	3	3	3	3	4	4	4	2
204	4	4	2	3	3	3	3	2	2	3	2	3	3	3	2	3	4	3	2	2
205	***1***	***1***	***0***	***1***	***0***	***0***	***1***	***1***	***1***	***0***	***1***	***1***	***1***	***0***	***0***	***1***	***1***	***1***	***2***	***2***
206	4	3	3	2	4	2	4	2	4	2	3	3	3	3	3	3	4	4	3	3
207	4	3	3	2	4	2	4	2	2	3	2	3	4	3	2	2	4	4	3	3
208	4	4	4	3	4	4	4	4	2	4	2	3	4	3	3	3	4	4	3	4
209	4	4	4	3	2	3	4	4	3	3	3	4	4	3	4	4	4	4	3	3
210	4	4	3	2	4	2	4	2	3	2	2	3	3	2	3	3	4	4	4	2
211	3	4	3	2	4	2	4	2	1	3	2	2	2	3	2	2	4	4	4	3
212	4	3	4	3	4	4	3	4	3	4	3	4	4	3	3	3	4	4	2	3
213	4	4	4	2	3	3	3	3	3	3	4	4	3	3	4	3	4	4	2	3
214	***4***	***4***	***2***	***2***	***3***	***2***	***4***	***2***	***2***	***3***	***2***	***2***	***1***	***2***	***1***	***1***	***4***	***4***	***3***	***2***
215	3	4	3	2	4	2	4	2	1	2	2	2	2	3	2	2	4	4	3	2
216	4	3	3	2	4	4	3	4	3	4	3	3	3	3	3	3	4	4	2	2
217	4	3	3	2	3	2	3	3	2	4	3	4	4	4	3	3	4	4	3	3
218	4	4	2	2	4	2	4	2	2	3	2	2	2	4	2	2	4	4	2	3
219	3	4	4	2	4	2	3	2	2	3	2	2	2	4	2	2	4	4	2	2
220	4	3	4	2	4	4	3	4	3	4	3	4	4	4	3	3	4	4	3	3

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Compound No	*C dubliensis* MYA646	*C glabrata* ATCC 90030	*C glabrata* WT-5	*C glabrata* 54640	*C krusei* ATCC 6258	*C krusei* CK25	*C tropicalis* ATCC 13803	*C tropicalis* 90/1362	*C tropicalis* 58660	*C parapsilosis* ATCC 22014	*C parapsilosis* 90/2224	*C albicans* SAP 1-3	*C albicans* SAP 4-6	*C albicans* ATCC 18804	*C albicans* ATCC 28367	*C albicans* 71929	*C albicans* 12	*C albicans* 194	*C kefyr* WT-1	*C guilliermondii* 192006
221	4	3	4	3	2	3	3	3	2	4	4	4	4	4	2	4	4	4	3	3
222	3	4	3	4	4	4	3	2	1	4	4	3	3	4	3	3	4	4	3	1
223	4	4	4	2	4	2	4	3	2	4	3	2	2	4	2	2	4	4	2	4
224	4	3	4	2	4	4	4	4	3	4	3	4	4	4	3	3	4	4	3	4
225	***1***	***0***	***1***	***2***	***1***	***1***	***1***	***1***	***1***	***1***	***1***	***1***	***1***	***3***	***2***	***2***	***1***	***1***	***1***	***1***
226	4	4	4	4	4	2	4	4	4	3	4	4	4	2	2	4	4	3	4	4
227	4	4	4	3	4	2	4	4	4	4	2	3	4	4	2	4	4	3	4	4
228	3	4	4	4	4	3	4	2	4	4	3	4	4	4	3	4	4	3	3	0
229	4	4	4	4	4	3	4	2	4	4	4	3	4	4	4	4	4	2	2	4
230	3	2	4	3	3	2	4	3	2	3	3	4	4	3	2	4	3	2	3	2
231	3	3	4	2	3	2	3	4	3	3	2	3	4	3	3	3	4	3	4	3
232	3	4	4	3	3	3	3	2	3	4	2	4	4	4	4	3	4	3	3	3
233	***3***	***1***	***0***	***3***	***2***	***2***	***2***	***1***	***2***	***2***	***1***	***2***	***1***	***3***	***4***	***2***	***1***	***1***	***2***	***2***
234	2	3	4	4	3	3	4	4	3	2	2	4	3	3	3	4	4	4	4	3
235	4	3	4	3	3	2	3	4	3	2	2	3	2	3	3	2	4	3	3	3
236	4	3	3	4	4	3	4	2	2	3	1	3	2	4	4	2	3	4	2	3
237	4	2	4	3	4	4	4	3	3	4	4	4	3	4	4	4	4	3	4	4
238	3	4	4	4	3	3	4	3	3	3	3	3	4	3	3	4	4	4	4	4
239	4	4	4	4	3	2	2	4	3	3	3	3	3	4	2	2	4	2	3	3
240	3	3	4	3	4	2	3	4	2	3	3	3	3	4	3	4	2	3	3	3

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Compound No	*C dubliensis* MYA646	*C glabrata* ATCC 90030	*C glabrata* WT-5	*C glabrata* 54640	*C krusei* ATCC 6258	*C krusei* CK25	*C tropicalis* ATCC 13803	*C tropicalis* 90/1362	*C tropicalis* 58660	*C parapsilosis* ATCC 22014	*C parapsilosis* 90/2224	*C albicans* SAP 1-3	*C albicans* SAP 4-6	*C albicans* ATCC 18804	*C albicans* ATCC 28367	*C albicans* 71929	*C albicans* 12	*C albicans* 194	*C kefyr* WT-1	*C guilliermondii* 192006
241	4	2	3	4	4	3	4	3	4	4	4	3	3	4	4	3	4	4	4	3
242	3	4	4	4	3	3	4	3	4	3	4	4	4	4	3	4	4	4	3	2
243	4	3	3	4	3	2	2	2	3	3	4	4	3	2	2	3	3	2	3	3
244	4	4	3	2	3	3	3	1	2	4	2	2	2	2	4	2	2	4	3	3
245	4	4	4	3	4	4	4	4	4	4	3	4	4	4	4	4	4	4	3	2
246	3	4	4	4	3	3	4	4	4	2	4	3	4	4	2	4	4	4	2	2
247	***4***	***3***	***3***	***3***	***2***	***1***	***1***	***2***	***2***	***3***	***1***	***3***	***3***	***3***	***2***	***1***	***4***	***3***	***2***	***1***
248	3	3	4	2	3	2	3	2	2	3	2	3	3	3	3	2	2	4	3	4
249	4	4	4	3	4	4	4	4	4	4	3	4	3	4	4	4	4	4	4	3
250	4	3	4	3	2	3	4	4	3	3	3	3	3	4	2	4	4	4	2	2
251	4	3	4	2	3	2	3	3	3	2	2	3	3	3	2	2	2	4	3	2
252	4	3	4	1	4	2	3	2	2	3	2	3	3	3	3	2	2	4	4	3
253	4	4	4	2	3	4	4	3	4	4	3	3	4	4	4	4	4	3	3	2
254	4	4	4	3	4	3	4	3	3	3	3	3	3	4	2	4	4	4	2	2
255	4	3	4	3	3	3	3	2	2	2	2	4	3	3	3	2	2	4	3	2
256	4	3	4	1	3	2	2	1	2	3	2	4	4	3	3	3	3	4	4	2
257	3	4	4	2	3	4	4	3	4	4	3	3	3	4	4	4	4	4	3	3
258	4	3	3	3	3	2	4	4	3	3	3	4	4	4	3	3	4	4	3	2
259	4	3	3	2	4	3	3	2	2	2	2	3	3	3	2	2	2	4	4	1
260	4	3	4	2	4	3	2	2	2	3	1	3	3	3	4	2	2	4	4	2

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Compound No	*C dubliensis* MYA646	*C glabrata* ATCC 90030	*C glabrata* WT-5	*C glabrata* 54640	*C krusei* ATCC 6258	*C krusei* CK25	*C tropicalis* ATCC 13803	*C tropicalis* 90/1362	*C tropicalis* 58660	*C parapsilosis* ATCC 22014	*C parapsilosis* 90/2224	*C albicans* SAP 1-3	*C albicans* SAP 4-6	*C albicans* ATCC 18804	*C albicans* ATCC 28367	*C albicans* 71929	*C albicans* 12	*C albicans* 194	*C kefyr* WT-1	*C guilliermondii* 192006
261	3	3	3	2	3	4	4	4	4	4	3	4	4	3	4	4	4	4	3	3
262	4	3	4	3	3	3	3	3	2	3	3	4	4	3	2	4	4	4	3	2
263	4	3	3	2	3	3	2	3	2	4	2	4	4	3	3	3	2	4	4	3
264	4	3	4	3	4	4	2	2	2	3	2	2	1	3	4	2	2	4	4	3
265	4	3	3	3	3	4	4	3	3	4	2	4	4	3	4	3	4	4	3	2
266	4	4	4	3	4	2	3	4	2	4	3	4	4	4	4	4	3	4	4	3
267	4	4	3	2	4	3	3	4	3	4	2	4	4	3	3	4	3	4	4	2
268	4	4	4	3	4	4	4	3	3	3	3	4	4	2	3	3	4	4	4	2
269	***4***	***1***	***0***	***1***	***1***	***1***	***1***	***1***	***2***	***1***	***1***	***4***	***3***	***4***	***2***	***3***	***4***	***3***	***3***	***1***
270	4	4	4	3	4	3	4	4	4	4	3	4	4	4	3	4	3	4	4	3
271	4	2	3	4	4	3	4	4	4	4	4	3	4	3	3	4	4	3	3	4
272	3	2	4	4	4	2	4	4	4	4	3	4	4	3	3	4	4	3	4	3
273	4	3	4	4	4	3	4	4	4	4	4	4	4	4	3	4	4	2	3	3
274	3	2	1	4	4	2	4	4	4	4	3	4	4	4	3	4	4	3	3	2
275	3	2	3	3	4	3	4	4	4	4	4	3	4	3	2	4	4	3	3	2
276	3	2	4	2	4	3	2	3	3	4	4	4	4	3	3	3	4	2	3	2
277	4	2	2	3	3	2	2	2	2	4	2	4	4	4	4	3	4	2	2	2
278	4	3	3	2	4	2	4	2	4	4	2	3	2	4	3	2	4	4	3	2
279	3	3	3	3	4	3	4	4	4	4	3	3	4	4	2	4	4	3	3	2
280	3	2	2	2	2	2	2	3	3	4	2	4	3	3	2	4	4	2	2	1

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Compound No	*C dubliensis* MYA646	*C glabrata* ATCC 90030	*C glabrata* WT-5	*C glabrata* 54640	*C krusei* ATCC 6258	*C krusei* CK25	*C tropicalis* ATCC 13803	*C tropicalis* 90/1362	*C tropicalis* 58660	*C parapsilosis* ATCC 22014	*C parapsilosis* 90/2224	*C albicans* SAP 1-3	*C albicans* SAP 4-6	*C albicans* ATCC 18804	*C albicans* ATCC 28367	*C albicans* 71929	*C albicans* 12	*C albicans* 194	*C kefyr* WT-1	*C guilliermondii* 192006
281	4	3	2	2	3	3	2	3	2	4	2	4	3	3	2	4	3	2	2	3
282	3	3	3	2	3	2	4	3	4	4	2	3	3	4	2	2	4	4	3	2
283	3	4	4	3	4	3	4	2	3	4	4	4	4	4	3	4	4	4	3	3
284	4	3	3	2	3	2	2	3	2	4	3	4	3	3	1	4	4	2	3	3
285	4	3	3	3	3	3	2	3	2	4	2	3	3	3	3	4	3	2	3	3
286	4	2	4	3	4	2	4	4	4	4	2	4	4	4	3	4	4	3	4	2
287	4	3	4	3	4	2	4	3	4	4	4	4	4	3	2	4	4	3	3	2
288	3	2	1	2	2	2	2	3	3	4	3	3	4	2	3	2	4	3	3	2
289	4	4	4	3	3	2	2	2	2	4	2	3	4	4	2	3	2	3	2	3
290	4	4	4	2	4	2	4	4	4	4	2	4	4	4	3	4	4	4	3	2
291	4	4	4	4	4	3	3	3	3	4	4	4	3	4	2	4	4	4	3	2
292	4	3	4	4	3	3	2	2	3	4	3	4	3	2	2	3	3	4	2	2
293	4	3	3	3	4	2	2	2	2	4	2	3	3	4	3	3	2	4	3	3
294	4	3	3	3	4	4	4	3	4	4	2	4	3	4	4	4	4	4	4	3
295	3	4	4	4	4	3	3	4	4	4	4	4	3	3	3	4	4	4	3	2
296	4	3	4	3	3	3	2	1	2	3	3	4	3	2	1	3	2	3	2	2
297	3	3	4	2	3	2	2	1	2	4	2	3	3	3	3	2	3	3	3	2
298	4	4	3	2	4	3	4	3	4	4	2	4	3	4	4	4	4	4	3	2
299	3	4	4	4	4	3	4	3	4	4	4	3	4	2	3	2	4	4	4	2
300	3	3	4	3	3	3	3	2	4	3	2	3	4	3	2	2	2	4	3	2

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Compound No	*C dubliensis* MYA646	*C glabrata* ATCC 90030	*C glabrata* WT-5	*C glabrata* 54640	*C krusei* ATCC 6258	*C krusei* CK25	*C tropicalis* ATCC 13803	*C tropicalis* 90/1362	*C tropicalis* 58660	*C parapsilosis* ATCC 22014	*C parapsilosis* 90/2224	*C albicans* SAP 1-3	*C albicans* SAP 4-6	*C albicans* ATCC 18804	*C albicans* ATCC 28367	*C albicans* 71929	*C albicans* 12	*C albicans* 194	*C kefyr* WT-1	*C guilliermondii* 192006
301	3	3	3	2	3	3	2	3	2	4	2	4	4	2	4	3	2	4	2	2
302	4	4	3	3	4	3	4	4	4	4	3	4	4	4	3	4	4	4	3	3
303	4	3	3	4	3	2	3	3	4	2	3	3	3	2	3	1	4	4	4	3
304	2	2	2	3	2	3	2	2	2	3	1	4	4	2	3	3	2	4	3	1
305	2	3	3	3	1	3	3	3	2	4	2	4	4	3	2	3	2	4	3	2
306	4	4	3	2	3	4	3	3	3	4	3	3	4	4	3	4	4	3	3	3
307	3	4	3	4	2	2	3	3	3	2	4	3	3	3	4	2	4	4	3	2
308	4	4	3	3	3	3	2	2	2	3	2	4	4	4	2	3	3	4	2	2
309	3	4	4	3	3	3	2	2	2	3	3	3	3	3	3	3	3	4	2	2
310	3	4	3	2	3	4	3	3	3	4	4	3	3	4	4	4	4	4	3	3
311	4	4	3	4	3	3	3	2	2	3	4	4	4	3	3	3	3	4	2	4
312	3	4	3	3	2	3	2	2	2	3	3	3	4	3	4	4	3	4	3	2
313	4	4	4	3	4	4	2	3	2	3	4	3	4	4	3	3	3	3	4	2
314	4	4	3	3	3	4	3	3	3	4	4	4	4	4	3	4	4	4	4	3
315	4	4	4	4	4	3	4	4	4	3	4	4	3	1	3	4	4	4	3	4
316	3	4	4	4	4	3	4	3	4	4	4	4	4	4	3	4	4	4	4	4
317	4	4	4	2	4	3	4	2	4	4	4	4	3	4	2	4	4	3	4	3
318	3	4	3	2	4	3	4	4	4	4	4	4	4	4	4	3	4	3	3	4
319	3	4	4	3	4	4	4	4	4	4	4	4	4	4	3	4	4	3	4	4
320	4	4	4	3	4	4	4	2	2	4	3	4	3	4	2	4	4	4	4	3

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Compound No	*C dubliensis* MYA646	*C glabrata* ATCC 90030	*C glabrata* WT-5	*C glabrata* 54640	*C krusei* ATCC 6258	*C krusei* CK25	*C tropicalis* ATCC 13803	*C tropicalis* 90/1362	*C tropicalis* 58660	*C parapsilosis* ATCC 22014	*C parapsilosis* 90/2224	*C albicans* SAP 1-3	*C albicans* SAP 4-6	*C albicans* ATCC 18804	*C albicans* ATCC 28367	*C albicans* 71929	*C albicans* 12	*C albicans* 194	*C kefyr* WT-1	*C guilliermondii* 192006
321	4	4	3	2	3	3	4	1	2	4	3	4	4	4	3	3	3	3	2	3
322	3	4	2	2	2	2	4	2	3	3	2	4	4	4	3	3	3	4	2	4
323	3	4	3	3	4	4	4	4	4	3	4	4	4	4	4	3	4	4	3	4
324	4	4	4	3	4	4	3	3	3	4	3	3	3	4	3	4	4	4	4	3
325	4	4	4	2	2	3	3	4	2	3	3	3	4	3	2	2	3	3	2	3
326	Insoluble in DMSO																			
327	3	4	3	4	4	3	4	4	4	4	4	4	3	4	3	3	4	4	4	4
328	4	4	4	4	4	4	4	3	3	4	3	3	4	3	3	4	4	3	3	3
329	4	4	3	3	3	3	3	1	2	4	2	3	4	3	2	3	4	4	4	3
330	***3***	***4***	***2***	***2***	***3***	***1***	***1***	***1***	***3***	***2***	***2***	***2***	***1***	***2***	***1***	***2***	***1***	***2***	***3***	***2***
331	4	4	4	2	4	3	4	3	4	4	4	3	3	4	3	4	4	4	4	4
332	4	4	3	2	3	4	4	4	3	4	4	4	4	3	4	4	4	4	3	3
333	4	4	3	3	2	3	3	2	2	4	3	4	3	3	2	2	4	4	2	3
334	3	4	3	4	3	2	2	2	3	3	2	3	4	4	2	3	3	4	4	3
335	4	4	4	2	4	3	4	4	4	4	4	3	4	4	3	4	4	4	3	4
336	4	4	2	4	4	3	4	4	3	3	4	4	3	3	4	2	4	4	2	2
337	4	4	3	3	3	2	3	3	2	3	3	3	4	3	2	3	3	4	3	3
338	4	4	2	4	2	4	2	2	3	3	2	3	4	2	2	4	2	4	3	4
339	4	3	4	2	4	4	4	4	4	4	3	4	3	4	3	4	4	4	4	4
340	4	4	3	4	4	3	4	4	3	4	3	3	3	3	4	4	4	4	3	3

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Compound No	*C dubliensis* MYA646	*C glabrata* ATCC 90030	*C glabrata* WT-5	*C glabrata* 54640	*C krusei* ATCC 6258	*C krusei* CK25	*C tropicalis* ATCC 13803	*C tropicalis* 90/1362	*C tropicalis* 58660	*C parapsilosis* ATCC 22014	*C parapsilosis* 90/2224	*C albicans* SAP 1-3	*C albicans* SAP 4-6	*C albicans* ATCC 18804	*C albicans* ATCC 28367	*C albicans* 71929	*C albicans* 12	*C albicans* 194	*C kefyr* WT-1	*C guilliermondii* 192006
341	4	4	3	3	2	3	3	3	1	3	3	4	4	2	2	3	4	4	3	3
342	3	4	3	2	3	4	2	2	2	3	2	3	4	1	2	3	2	4	3	4
343	4	4	4	2	4	4	4	3	4	4	3	4	4	4	3	4	4	4	4	4
344	4	4	2	4	4	3	4	3	2	4	3	3	4	3	3	4	4	4	3	3
345	3	4	3	3	2	2	2	2	1	3	2	3	3	3	2	2	3	4	2	1
346	3	4	4	2	2	4	2	2	2	2	2	2	3	2	2	2	2	4	3	3
347	4	4	4	2	4	4	4	3	4	4	3	3	3	4	3	4	4	4	4	4
348	4	4	4	4	2	2	3	2	2	4	3	4	3	1	3	3	4	4	2	1
349	3	4	4	4	2	2	2	2	2	3	2	3	3	2	2	2	2	4	3	2
350	Insoluble in DMSO																			
351	4	4	4	2	2	4	4	3	4	4	3	4	4	4	3	4	4	4	4	4
352	4	4	2	4	3	2	4	3	2	3	2	4	3	1	2	2	3	4	2	1
353	4	3	3	4	2	2	3	2	2	3	2	4	4	2	3	2	2	4	2	2
354	Insoluble in DMSO																			
355	4	4	4	3	3	4	3	4	4	3	3	3	3	4	3	4	3	4	3	4
356	3	4	3	4	4	2	4	2	2	4	3	4	4	3	3	1	3	4	3	2
357	***4***	***1***	***1***	***3***	***1***	***1***	***4***	***1***	***1***	***4***	***1***	***3***	***3***	***1***	***3***	***2***	***2***	***4***	***1***	***1***
358	4	4	4	4	3	3	4	3	3	4	3	4	3	3	4	3	4	4	3	3
359	4	4	4	3	3	4	4	4	4	3	3	3	4	4	2	4	4	4	4	4
360	3	0	3	4	2	3	3	4	4	3	2	4	4	4	3	3	4	4	4	2

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Compound No	*C dubliensis* MYA646	*C glabrata* ATCC 90030	*C glabrata* WT-5	*C glabrata* 54640	*C. krusei* ATCC 6258	*C krusei* CK25	*C tropicalis* ATCC 13803	*C tropicalis* 90/1362	*C tropicalis* 58660	*C parapsilosis* ATCC 22014	*C parapsilosis* 90/2224	*C albicans* SAP 1-3	*C albicans* SAP 4-6	*C albicans* ATCC 18804	*C albicans* ATCC 28367	*C albicans* 71929	*C albicans* 12	*C albicans* 194	*C kefyr* WT-1	*C guilliermondii* 192006
361	3	4	4	4	4	4	4	3	4	2	4	4	4	4	4	4	4	4	4	4
362	4	4	4	3	4	4	3	3	4	3	4	4	4	3	3	4	4	4	3	3
363	4	4	4	4	4	4	3	4	4	3	4	4	4	3	2	3	4	4	2	3
364	4	4	4	4	4	4	4	4	4	4	4	4	4	3	2	4	4	4	2	4
365	4	4	4	4	4	2	3	4	2	3	3	4	3	3	3	4	4	3	4	2
366	Insoluble in DMSO																			
367	4	4	4	2	4	4	2	1	3	4	2	3	4	1	2	2	4	4	3	2
368	Insoluble in DMSO																			
369	4	4	4	2	3	3	3	3	3	4	4	3	3	3	3	4	4	4	3	3
370	4	4	4	3	4	2		3	2	3	3	3	3	2	2	4	2	2	3	4
371	4	4	4	4	4	3	2	1	3	4	2	3	3	2	2	3	3	2	4	3
372	4	4	4	3	4	4	4	2	4	4	4	3	3	3	3	4	4	4	2	4
373	4	4	4	3	4	3	3	3	2	3	3	3	4	3	3	4	4	4	2	3
374	Insoluble in DMSO																			
375	4	4	4	2	3	3	3	2	3	4	3	3	3	2	3	4	3	3	3	3
376	Insoluble in DMSO																			
377	4	4	4	4	4	3	4	3	3	4	3	4	3	4	3	4	4	4	3	4
378	***3***	***3***	***1***	***3***	***4***	***3***	***1***	***1***	***1***	***2***	***1***	***3***	***3***	***2***	***2***	***4***	***4***	***2***	***2***	***3***
379	***4***	***4***	***2***	***2***	***1***	***1***	***2***	***2***	***2***	***2***	***2***	***4***	***4***	***1***	***2***	***3***	***2***	***3***	***2***	***2***
380	4	4	4	2	4	4	4	1	4	4	4	4	3	3	3	4	4	3	3	3

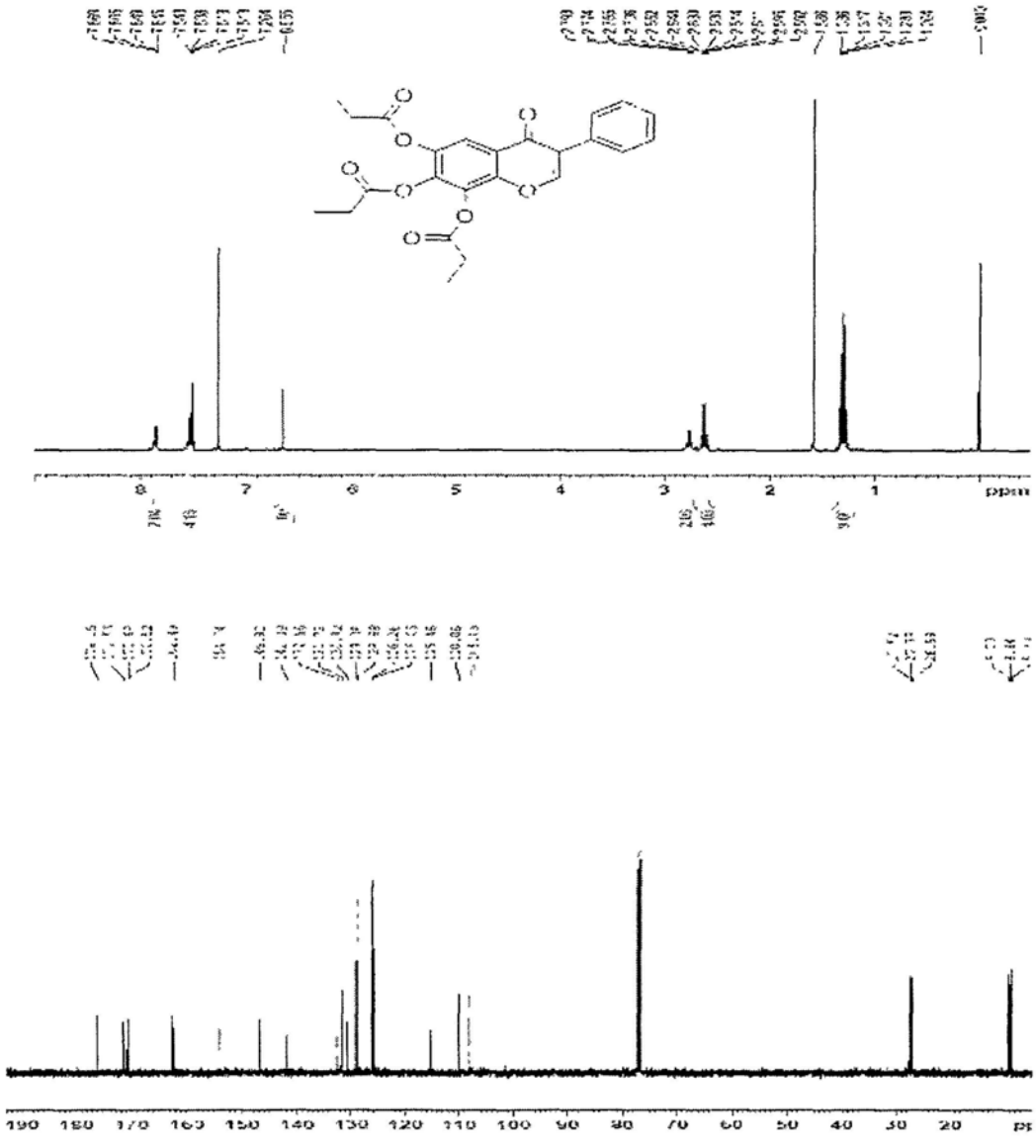
Continued

Compound No	*C dubliensis* MYA646	*C glabrata* ATCC 90030	*C glabrata* WT-5	*C glabrata* 54640	*C krusei* ATCC 6258	*C krusei* CK25	*C tropicalis* ATCC 13803	*C tropicalis* 90/1362	*C tropicalis* 58660	*C parapsilosis* ATCC 22014	*C parapsilosis* 90/2224	*C albicans* SAP 1-3	*C albicans* SAP 4-6	*C albicans* ATCC 18804	*C albicans* ATCC 28367	*C albicans* 71929	*C albicans* 12	*C albicans* 194	*C kefyr* WT-1	*C guilliermondii* 192006
381	4	3	4	3	4	3	3	3	3	3	3	4	3	4	2	4	4	4	3	3
382	4	4	4	3	3	3	2	2	2	2	2	4	4	2	3	4	3	3	3	3
383	4	4	3	2	2	4	2	3	2	3	3	4	4	2	2	3	3	4	3	2
384	4	4	4	2	4	4	4	4	4	4	3	4	4	4	3	4	4	3	4	4
385	4	4	4	4	4	3	4	3	4	4	3	3	4	4	4	4	4	4	3	3
386	4	4	4	3	3	2	2	2	2	2	2	4	3	2	2	3	2	3	2	2
387	4	3	4	2	2	3	2	2	2	3	3	3	3	3	3	2	2	4	3	3
388	4	4	4	2	4	4	4	4	4	4	4	4	4	4	4	4	4	3	4	4
389	4	4	4	4	3	4	4	3	2	4	3	4	4	2	3	4	3	4	3	4
390	4	4	3	2	2	2	1	2	1	3	2	3	3	2	2	2	2	4	2	4
391	3	3	3	2	2	3	2	1	2	2	2	4	3	4	3	1	2	4	2	4
392	4	4	4	2	4	4	4	3	4	4	4	3	4	4	3	4	4	4	4	4
393	4	4	4	4	4	3	3	3	3	4	3	4	3	3	3	2	3	4	3	4
394	4	4	4	3	3	2	2	2	2	3	3	4	4	3	2	2	2	4	2	4
395	4	4	4	2	2	2	2	3	2	1	2	4	3	4	3	4	2	4	2	4
396	4	4	3	2	3	4	4	3	4	3	3	4	4	4	3	3	3	4	3	4
397	4	4	4	4	3	3	3	2	3	4	3	4	4	3	4	2	3	4	3	4
398	3	4	4	2	3	2	2	1	2	3	3	3	3	4	3	2	2	4	2	4
399	3	3	3	2	2	3	2	2	2	2	2	4	3	4	3	3	2	4	2	4
400	4	3	4	3	3	4	4	4	4	3	4	4	3	4	3	4	4	4	4	4

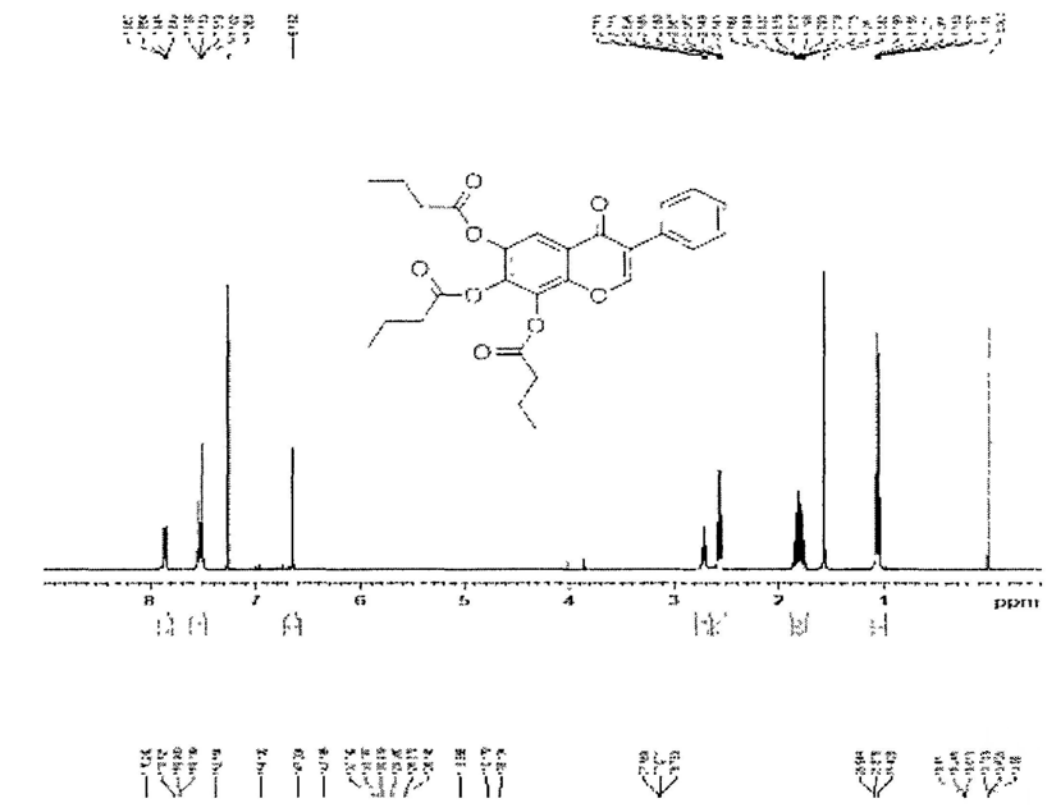
The screening was based on the standard method of M27-A, CLSI, with the final concentration of compound at 20 μM. The number was representive of the antifungal activity of compound. The smaller the number, the more potent the compound in its antifungal activity is.

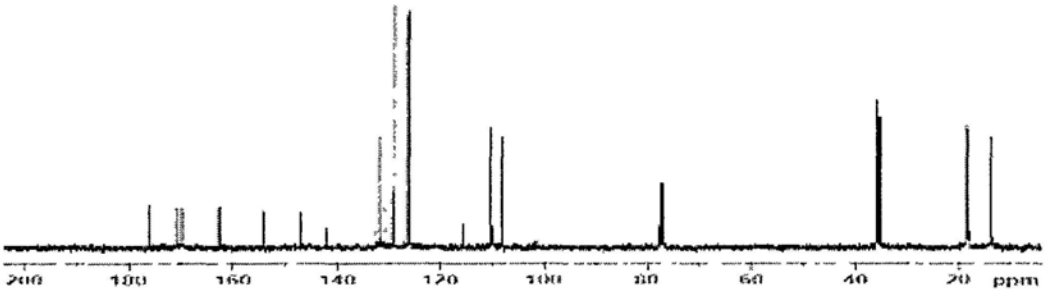
Appendix Ⅱ NMR data of baicalein derivatives

^{1}H and $^{13}C\{^{1}H\}$ NMR Spectra of 5,6,7-tri-*o*-propyl-baicalein in $CDCl_3$

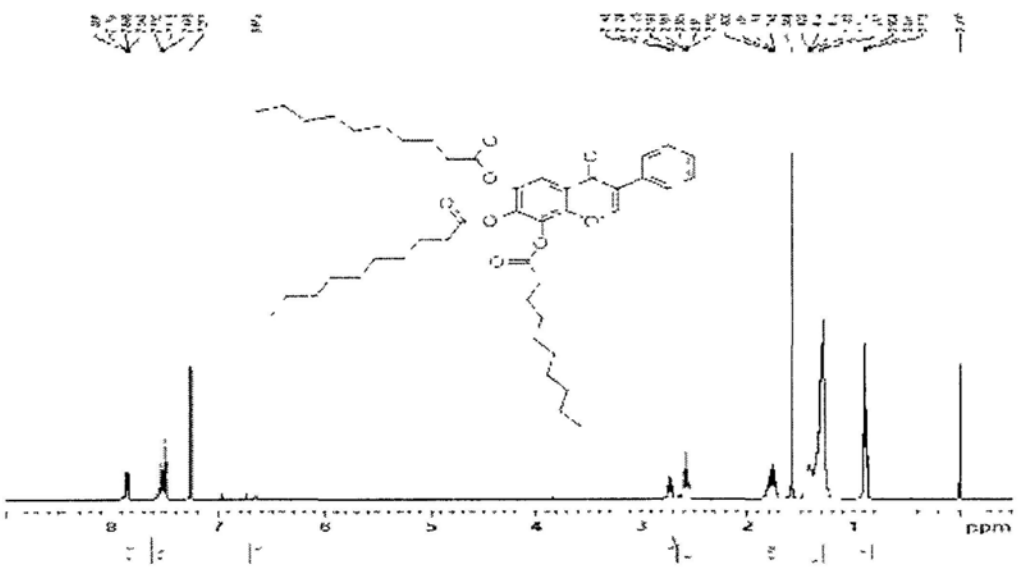


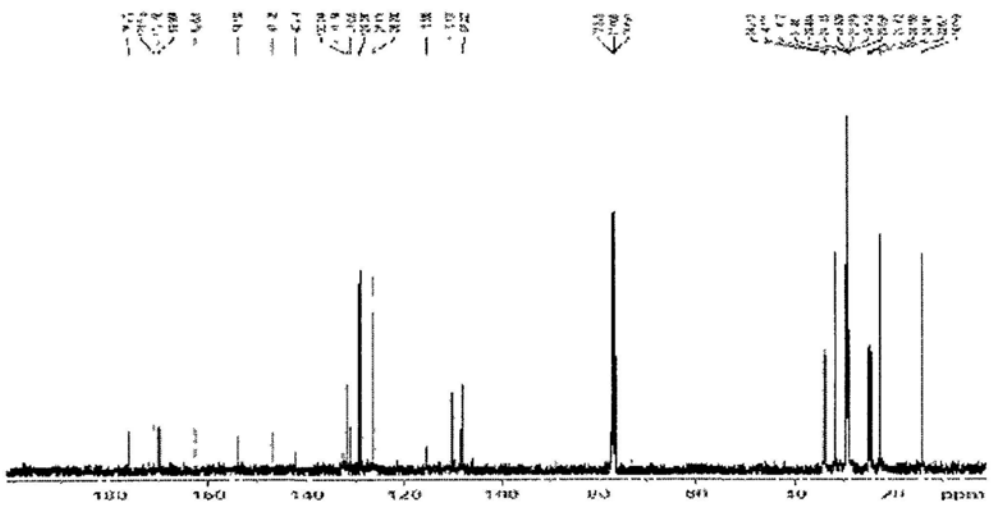
^{1}H and $^{13}C\{^{1}H\}$ NMR Spectra of 5,6,7-tri-*o*-butyl-baicalein in $CDCl_3$





^{1}H and $^{13}C\{^{1}H\}$ NMR Spectra of 5,6,7-tri-*o*-decanoyl-baicalein in $CDCl_3$





^{1}H and $^{13}C\{^{1}H\}$ NMR Spectra of 5,6,7-tri-*o*-benzoyl-baicalein in $CDCl_3$